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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q		A2	(11) International Publication Number: WO 00/11204 (43) International Publication Date: 2 March 2000 (02.03.00)
(21) International Application Number: PCT/US99/18973 (22) International Filing Date: 18 August 1999 (18.08.99) (30) Priority Data: 60/097,334 18 August 1998 (18.08.98) US 60/138,493 9 June 1999 (09.06.99) US 60/138,426 9 June 1999 (09.06.99) US 60/138,494 9 June 1999 (09.06.99) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications US 60/097,334 (CIP) Filed on 18 August 1998 (18.08.98) US 60/138,426 (CIP) Filed on 9 June 1999 (09.06.99) US 60/138,493 (CIP) Filed on 9 June 1999 (09.06.99) US 60/138,494 (CIP) Filed on 9 June 1999 (09.06.99) (71) Applicant (for all designated States except US): THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only): WORLEY, Paul, F. [US/US]; 17 Blythewood Road, Baltimore, MD 21210 (US). TU, Jian, Cheng [CN/US]; 201 C Donnybrook Lane, Towson, MD 21286 (US). XIAO, Bo [CN/US]; The Johns Hopkins University School of Science, Department of Neuroscience, 720 Rutland Avenue, Baltimore, MD 21205 (US). LEAHY, Daniel [US/US]; 3805 Fenchurch Road, Baltimore, MD 21218 (US). BENEKEN, Jutta [DE/US]; 8 Charles Plaza #406, Baltimore, MD 21201 (US). LANAHAN, Anthony, A. [US/US]; 15 Dendron Court, Baltimore, MD 21234 (US). (74) Agent: HAILE, Lisa, A.; Gray Cary Ware & Friedenrich LLP, Suite 1600, 4365 Executive Drive, San Diego, CA 92121-2189 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: HOMER INTERACTING PROTEINS			
(57) Abstract A method is provided for identifying a compound that modulates a cellular response associated with Homer and mediated by a cell-surface or an intracellular receptor. A method is further provided for identifying a compound that modulates receptor activated calcium mobilization associated with Homer. A method is provided for identifying a compound that inhibits Homer protein activity based on the crystal structure coordinates of Homer protein binding domain. A method is also provided for identifying a compound that affects the formation of cell surface receptors into clusters. Also provided are nucleic acids encoding Homer proteins as well as Homer proteins, and Homer interacting proteins.			

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HOMER INTERACTING PROTEINS

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STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with Government support under Grant No. RO1 DA10309, RO1 DA11742 and KO2 MH01152, awarded by the National Institutes of Health. The government may have certain rights in the invention.

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FIELD OF THE INVENTION

The present invention relates generally to protein-protein interactions and more specifically to molecules involved in mediating receptor-activated or ion channel-mediated intracellular calcium mobilization or concentration.

15

BACKGROUND OF THE INVENTION

The mature central nervous system exhibits the capacity to alter cellular interactions as a function of the activity of specific neuronal circuits. This capacity is believed to underlie learning and memory storage, age-related memory loss, tolerance to and dependence on drugs of abuse, recovery from brain injury, epilepsy as well as aspects of postnatal development of the brain (Schatz, C., *Neuron*, 5:745, 1990). Currently, the role of activity-dependent synaptic plasticity is best understood in the context of learning and memory. Cellular mechanisms underlying activity-dependent plasticity are known to be initiated by rapid, transmitter-induced changes in membrane conductance properties and activation of intracellular signaling pathways (Bliss and Collingridge, *Nature*, 361:31, 1993). Several lines of evidence also indicate a role for rapid synthesis of mRNA and protein in long-term neuroplasticity. For example, classical studies of learning and memory demonstrate a requirement for protein synthesis in long-term, but not short-term memory (Flexner, *et al.*, *Science*, 141:57, 1963; Agranoff, B., *Basic*

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Neurochemistry, 3rd Edition, 1981; Davis and Squire, *Physiol. Bull.*, 96:518, 1984), and long-term enhancement of synaptic connectivity, studied in cultured invertebrate neurons (Montarolo, *et al.*, *Science*, 234:1249, 1986; Bailey, *et al.*, *Neuron*, 2:749, 1992) or in the rodent hippocampus (Frey, *et al.*, *Science*, 260:1661, 1993; Nguyen, *et al.*, *Science*,
5 265:1104, 1994), is blocked by inhibitors of either RNA or protein synthesis. Importantly, inhibitors of macromolecular synthesis are most effective when administered during a brief time window surrounding the conditioning stimulus indicating a special requirement for molecules that are rapidly induced (Goelet, *et al.*, *Nature*, 322:419, 1986).

10 Immediate early genes (IEGs) are rapidly induced in neurons by neurotransmitter stimulation and synaptic activity and are hypothesized to be part of the macromolecular response required for long-term plasticity (Goelet, *et al.*, *supra*; Sheng and Greenberg, *Neuron*, 4:477, 1990; Silva and Giese, *Neurobiology*, 4:413, 1994). To identify cellular mechanisms that may contribute to long-term plasticity in the vertebrate brain,
15 differential cloning techniques have been used to identify genes that are rapidly induced by depolarizing stimuli (Nedivi, *et al.*, *Nature*, 363:713, 1993; Qian, *et al.*, *Nature*, 361:453, 1993; Yamagata, *et al.*, *Neuron*, 11:371, 1993; Yamagata, *et al.*, *Learning and Memory* 1:140, 1994; Yamagata, *et al.*, *Journal of Biological Chemistry*, 269:16333, 1994; Andreasson and Worley, *Neuroscience*, 69:781, 1995; Lyford, *et al.*, *Neuron*,
20 14:433, 1995). In contrast to the earlier focus on transcription factors, many of the newly characterized IEGs represent molecules that can directly modify the function of cells and include growth factors (Nedivi, *et al.*, *supra*; Andreasson and Worley, *supra*), secreted enzymes that can modify the extracellular matrix, such as tissue plasminogen activator (Qian, *et al.*, *supra*), enzymes involved in intracellular signaling, such as prostaglandin
25 synthase (Yamagata, *et al.*, *supra*), and a novel homolog of H-Ras, termed Rheb (Yamagata, *et al.*, *supra*), as well as a novel cytoskeleton-associated protein, termed Arc (Lyford, *et al.*, *supra*). The remarkable functional diversity of this set of rapid response genes is representative of the repertoire of cellular mechanisms that are likely to contribute to activity-dependent neuronal plasticity.

Pharmaceutical agents often act by modulating signaling between cells or within cells. For example, Prozac alters the reuptake of the neurotransmitter serotonin and enhances aspects of its signaling function in brain. Nonsteroidal antiinflammatory drugs (NSAIDs) act by inhibiting the activity of cyclooxygenase enzyme, which is involved in the signaling pathways of inflammation. Viagra modifies the intracellular guanylate cyclase response to autonomic neurotransmitters in erectile tissues. These, and other precedent setting pharmaceuticals, validate the notion that specific signaling pathways may be targeted for therapeutic development.

Cellular mechanisms that modify important intracellular signals can involve changes in intracellular calcium. This type of mechanism is used in brain neurons to adapt to changes in intercellular signaling, and is demonstrated to exert powerful effects on cellular responses induced by glutamate. Similar, though distinct, cellular mechanism may be used to modulate intracellular calcium signals in other tissues including heart, lung, liver and skeletal muscle. Compounds that can modify this mechanism can modulate natural transmitter signals and may exert therapeutic effects.

Classical studies demonstrated that activation of receptors on the cell surface evoke changes in the level of specific, diffusable molecules inside the cell. The regulated production of these molecules serves to signal events happening at the membrane surface to intracellular receptors and are therefore termed second messenger signaling pathways. Major second messenger pathways include the phosphoinositide pathway, which regulates intracellular calcium; the adenylate cyclase pathway, which regulates levels of cyclic AMP; the guanylate cyclase pathway, which regulates levels of cGMP; and the nitric oxide pathway which regulates NO.

The regulated release of intracellular calcium is essential to the function of all tissues. Each tissue possesses a distinct physiology that is dependent on receptor/transmitter-regulated release of intracellular calcium. For example, synaptic function is modulated in brain neurons by glutamate receptor regulated release of intracellular calcium. Contractility of cardiac and smooth muscle is also regulated by intracellular calcium. Recent reviews of the role of calcium signaling in cellular responses include: Berridge, *Nature* **386**:759 (1997); Berridge, *J. Physiol. (London)* **499**:291 (1997); Bootman *et al.*, *Cell* **91**:367 (1997).

Recent studies demonstrate that molecules that function together in signaling networks are frequently clustered together in macromolecular complexes. For example, components of the MAP kinase pathway form a complex of cytosolic kinases with their specific substrates (Davis, *Mol. Reprod. Dev.* 42:459 (1995)).

5 Similarly, proteins such as AKAP function as scaffolds for specific kinases and their substrates (Lester and Scott, *Recent Prog. Horm. Res.* 52:409 (1997)). Recently, a multi-PDZ containing protein was identified in *Drosophila* (termed InaD) that couples the membrane-associated, light-activated ion channel with its effector enzymes (Tsunoda *et al.*, *Nature* 388:243 (1997)). The biochemical consequence of this

10 clustering is that the local concentrations of molecules that convey the signals between proteins are as high as possible. Consequently, signaling takes place efficiently. The clustering activity of these proteins is essential to normal function of the signaling cascade (Lester and Scott, *supra* 1997; Tsunoda *et al.*, *supra* 1997). Accordingly, Accordingly, agents that alter these signaling complexes will modify the

15 response due to transmitter or other form of cellular stimulation in a way that mimics more classical receptor agonists or antagonists. For example, a metabotropic glutamate receptor signaling may be blocked either at the receptor by conventional receptor antagonists or by uncoupling the metabotropic receptor from its intracellular IP3 receptor by agents that block the cross-linking activity of Homer family proteins.

20 The identification of molecules regulating the aggregation of neurotransmitter receptors at synapses is central to understanding the mechanisms of neural development, synaptic plasticity and learning. The most well characterized model for the synaptic aggregation of ionotropic receptors is the neuromuscular junction. Early work showed that contact between the axon of a motor neuron and the surface of a myotube rapidly

25 triggers the accumulation of preexisting surface acetylcholine receptors (Anderson and Cohen, *J Physiol* 268:757-773, 1977; Frank and Fischbach, *J Cell Biol* 83:143-158, 1979). Subsequent work has shown that agrin, a complex glycoprotein secreted by the presynaptic terminal, activates a postsynaptic signal transduction cascade (reviewed by Colledge and Froehner, *Curr Opin Neurobiol* 8:357-63, 1998), that leads to receptor

30 clustering by the membrane associated protein rapsyn.

SUMMARY OF THE INVENTION

Homer proteins, the products of neuronal immediate early genes, selectively bind the carboxy-termini of certain cell-surface receptors (*e.g.*, group 1 metabotropic receptors), certain intracellular receptors and binding proteins (*e.g.*, inositol trisphosphate receptors, ryanodine receptor, Shank proteins, I42). Many forms of Homer proteins contain a "coiled-coil" structure in the carboxy-terminal domain which mediates homo- and heteromultimerization between Homer proteins. The present invention is based on the seminal discovery that Homer plays a significant role in mediating receptor-activated calcium mobilization from internal stores and that Homer proteins regulate aspects of receptor clustering

In one embodiment, a method is provided for identifying a compound that modulates a cellular response mediated by a cell-surface receptor. The method includes incubating a test compound and a cell expressing a cell-surface receptor and a Homer protein under conditions sufficient to permit the compound to interact with the cell, and exposing the cell to a cell-surface receptor ligand. A cellular response to the ligand by the cell incubated with the compound is compared with a cellular response of the cell not incubated with the compound wherein a difference in cellular response identify a compound that modulates a Homer-associated cellular response.

In another embodiment, a method is provided for identifying a compound that modulates a cellular response mediated by an intracellular receptor. The method includes incubating the compound, and a cell expressing an intracellular receptor and a Homer protein under conditions sufficient to permit the compound to interact with the cell and exposing the cell to conditions that activate the intracellular receptor. A cellular response by a cell incubated with the compound is compared with a cellular response of a cell not incubated with the compound wherein a difference in a cellular response identifies a compound that modulates a Homer-associated cellular response.

In yet another embodiment, a method is provided for identifying a compound that modulates receptor activated calcium mobilization in a cell. The method includes incubating the compound and a cell expressing a Homer protein under conditions sufficient to permit the compound to interact with the cell and exposing the cell to

conditions sufficient to activate calcium mobilization. The receptor-activated calcium mobilization of a cell incubated with said the compound is compared with the receptor-activated calcium mobilization of a cell not incubated with the compound wherein a difference in calcium mobilization is indicative of an effect of the compound on Homer-associated calcium mobilization.

In another embodiment, a method is provided for modulating receptor-mediated calcium mobilization. The method includes exposing a cell expressing Homer protein to a compound in a sufficient amount to modulate the calcium mobilization that typically occurs when a cell is exposed to an amount of ligand sufficient to activate an intercellular signaling pathway that includes Homer protein.

In another embodiment, a method is provided for identifying a compound that inhibits Homer protein activity. The method includes identifying an inhibitor of Homer binding or crosslinking activity and identifying an inhibitor of Homer protein activity that forms covalent or non-covalent bonds with amino acids in a Homer protein binding site, based upon the crystal structure coordinates of Homer protein binding domain, and synthesizing the inhibitor.

In one embodiment, a method is provided for identifying a compound that affects the formation of cell surface receptors into clusters. The method includes incubating the compound and a cell expressing a Homer protein and a Homer interacting protein, e.g., a Shank protein, under conditions sufficient to allow the compound to interact with the cell and determining the effect of the compound on the formation of cell-surface receptors into clusters. The formation of cell-surface receptors into clusters of a cell contacted with the compound is compared to the formation of cell-surface receptors into clusters of a cell not contacted with the compound, wherein a difference in the formation of clusters is indicative of a compound that affects formation of cell surface receptors into clusters.

In another embodiment, a method is provided for treating a disorder associated with glutamate receptors, including metabotropic and NMDA-type glutamate receptors, in a subject. The method includes administering to a subject in need, a

therapeutically effective amount of a compound that modulates Homer protein activity.

In another embodiment, a method is provided for treating a disorder associated with Homer protein activity including administering to a subject in need a therapeutically effective amount of a compound that modulates Homer protein activity. The compound may be identified by a method of the invention described herein.

In another embodiment, there is provided an isolated nucleic acid encoding Homer protein 1b, having the nucleotide sequence as set forth in SEQ ID NO:3 as well as an isolated Homer protein having substantially the same amino acid sequence as set forth in SEQ ID NO:4.

In another embodiment, there is provided an isolated nucleic acid encoding Homer protein 1c, having the nucleotide sequence as set forth in SEQ ID NO:5 as well as an isolated Homer protein having substantially the same amino acid sequence as set forth in SEQ ID NO:6.

In another embodiment, there is provided an isolated nucleic acid encoding Homer protein 2a, having the nucleotide sequence as set forth in SEQ ID NO:7 as well as an isolated Homer protein having substantially the same amino acid sequence as set forth in SEQ ID NO:8.

In another embodiment, there is provided an isolated nucleic acid encoding Homer protein 2b, having the nucleotide sequence as set forth in SEQ ID NO:9 as well as an isolated Homer protein having substantially the same amino acid sequence as set forth in SEQ ID NO:10.

In another embodiment, there is provided an isolated nucleic acid encoding Homer protein 3, having the nucleotide sequence as set forth in SEQ ID NO:11 as well as an isolated Homer protein having substantially the same amino acid sequence as set forth in SEQ ID NO:12.

In another embodiment, there is provided an isolated peptide having the amino acid sequence set forth in SEQ ID NO:13 an isolated peptide having the amino acid sequence set forth in SEQ ID NO:14.

5 In yet another embodiment, there is provided an isolated nucleic acid encoding Homer Interacting Protein, having the nucleotide sequence as set forth in SEQ ID NO:15 or 17 with a deduced amino acid sequence as set forth in SEQ ID NO:16 or 18, respectively.

In another embodiment, there is provided an isolated Homer Interacting Protein having substantially the same amino acid sequence as set forth in SEQ ID
10 NO:19.

In another embodiment, there is provided an isolated Homer Interacting Protein having substantially the same amino acid sequence as set forth in SEQ ID NO:20.

15 In yet a further embodiment, there is provided a substantially purified polypeptide containing a proline rich region that is specifically capable of specifically binding to polypeptides of the Homer family.

In still another embodiment, there is provided a transgenic non-human animal having a transgene that expresses a Homer protein, e.g., Homer 1a, chromosomally integrated into the germ cells of the animal.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic Representation of EVH1 Domain-containing Proteins. EVH1 domains are found at or near the N-termini of Homer, Ena, Mena, VASP, and WASP proteins. Homer 1b/2/3 encode a CC domain which mediates multimerization
25 between various Homer proteins. In ENA, Mena, VASP, WASP, and N-WASP, the EVH1 domain is followed by a central proline rich region of variable length. The proteins are drawn to the scale shown, and the respective amino acid lengths are shown at the right.

Figure 2. Structure-Based Alignment of EVH1, PH, and PTB Domain Sequences. A structure-based sequence alignment between EVH1 domains and the β -spectrin PH domain and the IRS-1 PTB domain is shown. Species are indicated by Rn (rat), Hs (human), Mm (mouse), and Dm (*Drosophila*). Elements of the Homer EVH1 domain secondary structure are represented by arrows (β -strands), cylinders (α -helices), and lines (coils). Conserved residues (among EVH1 domains) are highlighted. The fractional solvent accessibility (FAS) of each residue in Homer 1a is indicated by ovals. Filled ovals = $0 \leq \text{FAS} \leq 0.1$ (buried); shaded ovals = $0.1 < \text{FAS} \leq 0.4$ (partially accessible); open ovals = $\text{FAS} > 0.4$. Mutations in the EVH1 domain of the WASP gene are indicated in lower case letters below the WASP amino acid sequence. Mutations that are associated with the severe WAS phenotype are shown in bold letters (Zhu et al, 1997). Sites mutated to more than one residue are indicated by asterisks. Bold asterisks indicate residues that, when mutated, affect the interaction of WASP with WIP (Stewart et al., 1999). Residues of Homer, β -spectrin, and IRS-1 that align well following structural superposition and were used to calculate rms differences in $\text{C}\alpha$ positions between these domains are underlined in the IRS-1 sequence. Gaps are indicated by dashes while continued sequences at amino- and carboxy-termini are indicated by periods. Residue numbering for Homer 1a is shown above its amino acid sequence. The number of the last included residue of each protein is shown at the end of each row.

Figure 3. Ribbon Diagram of the Homer 1a EVH1 Domain. The amino and carboxy termini are indicated, and elements of secondary structure are labeled to correspond to homologous structures in PH and PTB domains. An additional short region of β -strand between $\beta 1$ and $\beta 2$ has been labeled βi .

Figure 4. Structural Comparison of EVH1, PH, and PTB Domains. Ribbon diagrams (A)-(C) and surface representations (D)-(F) of the Homer 1 EVH1, β -spectrin PH, and IRS-1 PTB domains, respectively, are shown. All molecules are shown in a similar orientation, which is rotated about 45° about the vertical axis from orientations shown in Figure 3. The β -spectrin PH domain is shown with bound inositol trisphosphate (Hyvonen et al, 1995). The IRS-1 domain is shown complexed

to a phosphotyrosine-containing peptide derived from the insulin receptor (Eck et al., 1996).

Figure 5. Versatile Ligand Recognition by PH-Like Domain. Stereodiagram of a backbone trace of Homer 1 EVH1 domain showing the relative positions of IP3 bound by the β -spectrin and PLC- δ PH domains, as well as the peptide ligands for the IRS-1 and Numb PTB domains is shown. The orientations of the EVH1 domain is similar to that in Figure 4. Ligand positions were determined by superimposing the backbone traces of the EVH1, PH and PTB domains in the program) (Jones et al., 1991).

Figure 6. Mapping of WAS-Causing and Homer Binding Mutations on the EVH1 Surface. (A) and (B) Surface representations of the Homer1 EVH1 domain with sites homologous to positions of WASP mutations (in parentheses) colored according to solvent accessibility. Solvent exposed residues are shown in magenta, and buried or partially buried residues are shown in blue. Residue assignments are based on the sequence shown in Figure 2. WASP EVH1 mutations are listed in Table 2. Surface representations of Homer 1 EVH1 domain showing the location of residues targeted by site-directed mutagenesis. Mutations that disrupt binding of Homer EVH1 to ligands in an in vitro binding assay are shown in red, while those that have no effect on binding are shown in light blue (see Table 3). The orientation of the EVH1 domain in panels A and C is identical to that in Figure 4A and D. In panels B and D, the molecule is rotated about 180 degrees about the vertical axis.

Figures 7 through 47 are described in the following table.

Figure:s Homer Family Proteins and Homer Interacting Proteins

FIGURE	SEQ ID No.	Sequence
	1	Human Homer 1a (nucleic acid)
7	2	Human Homer 1a (amino acid)
8	3	Human Homer 1b (nucleic acid)
9	4	Human Homer 1b (amino acid)
	5	Homer 1c (nucleic acid)
	6	Homer 1c (amino acid)
10	7	Human Homer 2a (nucleic acid)
11	8	Human Homer 2a (amino acid)
12	9	Human Homer 2b (nucleic acid)
13	10	Human Homer 2b (amino acid)
14	11	Human Homer 3 (nucleic acid)
15	12	Human Homer 3 (amino acid)
16	13	peptide binding—core region: PPXXFR
17	14	peptide binding—extended region: ALTPSPFRD
18	15	Homer interacting protein: rat I30 (nucleic acid)
19	16	Homer interacting protein: rat I30 (amino acid)
20	17	Homer interacting protein: rat I42 (nucleic acid)
21	18	Homer interacting protein: rat I42 (amino acid)
22	19	Homer interacting protein: human I30 (nucleic acid)
23	20	Homer interacting protein: human I30 (amino acid)
24	21	Homer interacting protein: human I42 (nucleic acid)
25	22	Homer interactin protein: human I42 (amino acid)
26	23	Mouse Homer 1a (nucleic acid)
27	24	Mouse Homer 1a (amino acid)
28	25	Mouse Homer 1b (nucleic acid)
29	26	Mouse Homer 1b (amino acid)
30	27	Mouse Homer 2a (nucleic acid)

31	28	Mouse Homer 2a (amino acid)
32	29	Mouse Homer 2b (nucleic acid)
33	30	Mouse Homer 2b (amino acid)
34	31	Mouse Homer 3 (nucleic acid)
35	32	Mouse Homer 3 (amino acid)
36	33	Rat Homer 1a (nucleic acid)
37	34	Rat Homer 1a (amino acid)
38	35	Rat Homer 1b (nucleic acid)
39	36	Rat Homer 1b (amino acid)
40	37	Rat Homer 1c (nucleic acid)
41	38	Rat Homer 1c (amino acid)
42	39	Rat Shank 3a (nucleic acid)
43	40	Rat Shank 3a (amino acid)
44	41	Human Homer 3a (nucleic acid)
45	42	Human Homer 3a (amino acid)
46	43	Rat INADL partial nucleic acid sequence
47	44	Rat INADL partial amino acid sequence

DETAILED DESCRIPTION OF THE INVENTION

Homer represents a family of proteins that selectively binds the carboxy-terminus of group 1 metabotropic receptors and is enriched at excitatory synapses (Brakeman *et al.*, 1977). In the adult brain, Homer is rapidly and transiently induced
5 by physiological synaptic stimuli that evoke ion-term potentiation in the hippocampus (Brakeman *et al.*, 1997; Kato *et al.*, 1997), and is also induced in the striatum by dopaminetic drugs of addiction (Brakeman *et al.*, 1997). The first Homer gene identified, now termed Homer 1a (Brakeman *et al.*, *Nature* 386:2284-288 (1997); GenBank Accession No. U92079), is a member of a family of closely related Homer
10 proteins that are constitutively expressed in brain (Kato *et al.*, 1998; Sun *et al.*, 1998; Xiao *et al.*, 1998). There are now three mammalian genes identified and at least six distinct transcripts expressed in brain (Xiao *et al.*, 1998). All Homer family members, including Homer 1a, contain an amino-terminal region of about 110 amino acids that binds metabotropic glutamate receptors 1a and 5 (mGluR1a and mGluR5) (Xiao *et al.*,
15 *et al.*, 1998). The region of Homer that interacts with mGluR1a or 5 is termed "EVH1 domain", based on homology to similar domains in a family of proteins that include *Drosophila* Enabled (Gertler *et al.*, 1996), mammalian VASP (Haffner *et al.*, 1995) and the Wescott-Aldridge protein (WASP) (Ponting and Phillips, 1997; Symons *et al.*, 1996). The EVH1 domain of Homer is conserved at a level of about 80% between
20 *Drosophila*, rodent and human (Xiao *et al.*, 1998) The Homer family EVH1 domain also can bind to intracellular receptors such as the inositol trisphosphate receptor and dyamin III. Binding of Homer proteins in the EVH1 region is mediated by an amino acid sequence motif that is rich in proline residues.

To explore the proline-rich motif and its role in Homer interactions, a deletion
25 mutation strategy was used. A 50-amino acid deletion at the carboxy-terminal end of mGluR5 destroyed binding to Homer. By contrast, a 41 amino acid deletion of mGluR5 retained full binding activity. The intervening sequence is proline rich and shares sequence similarity with the previously described SH3 ligand sequence (Yu, 1994). A series of point mutants based on the known structure-function relationship
30 for SH3 ligands was prepared and binding assays confirmed general characteristics of SH3 ligand binding, but also demonstrated that that the Homer binding site is distinct

in the positioning of critical amino acids (Tu *et al.*, 1998). A consensus for binding was determined to be PPXXFR, consistent with the observation that mutation of either of the proline residues or the phenylalanine, or a change in their relative position, interrupted binding. The arginine in the last position was preferred over
5 other tested amino acids, but is not essential. Mutations were identically effective in interrupting binding to each of the Homer family members including Homer 1a, 1b/c, 2a/b, 3 and an EVH1 fragment (110 amino acids) of Homer 1. Thus, it was concluded that the interaction with mGluR5 was mediated by the Homer EVH1 domain.

To further explore Homer binding, mutations of mGluR5 were tested using a
10 250 amino acid carboxy-terminal fragment of the receptor, which had an identical effect on binding when placed in the full length mGluR5 protein (Tu *et al.*, 1998). This exquisite sensitivity of Homer binding to changes in single amino acids within the Homer-ligand site was confirmed in other Homer-interacting proteins including mGluR1a (Tu *et al.*, 1998), Shank (Tu *et al.*, 1999), and I42 (see below). To further
15 confirm that the interaction was mediated by a direct interaction at the Homer-ligand site (as opposed to a secondary allosteric effect on a remote binding site), synthetic 10-mer peptides with either the wild type, or F-to-R mutation were prepared. The wild type peptide blocked binding of mGluR1a or mGluR5 to each of the Homer family members (Tu *et al.*, 1998). Approximately half of the binding was blocked at a
20 peptide concentration of 3.4 micromolar. By contrast, the F-to-R mutant peptide did not alter binding at concentrations as high as 340 micromolar.

Most forms of Homer protein encode a carboxy-terminal domain with a "coiled-coil" structure. This coiled-coil domain mediates homo- and heteromultimerization between Homer proteins (Kato *et al.*, 1998; Xiao *et al.*, 1998)
25 and such multimers can be identified in normal brain tissue (Xiao *et al.*, 1998). Homer proteins are enriched in brain tissue fractions from postsynaptic densities and are localized at the ultrastructural level to postsynaptic densities. Homer 1a differs from the other members of the Homer family in that Homer 1a is not constitutively expressed and it does not contain a carboxy terminal coiled-coil domain.
30 Experimental data showing that Homer proteins interact with cell-surface receptors

and with intracellular receptors, and form multimeric complexes with other Homer proteins indicates an important role for Homer proteins in intracellular signaling.

An exemplary polynucleotide encoding a Homer protein is set forth as SEQ ID NO: 1. The term "polynucleotide", "nucleic acid", "nucleic acid sequence", or
5 "nucleic acid molecule" refers to a polymeric form of nucleotides at least 10 bases in length. By "isolated polynucleotide" is meant a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore
10 includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. A
15 polynucleotide encoding Homer includes "degenerate variants", sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of a polypeptide encoded by the nucleotide sequence of SEQ ID NO: 1 is functionally
20 unchanged.

A nucleic acid molecule encoding Homer includes sequences encoding functional Homer polypeptides as well as functional fragments thereof. As used herein, the term "functional polypeptide" refers to a polypeptide which possesses biological function or activity which is identified through a defined functional assay
25 (e.g., EXAMPLE 3), and which is associated with a particular biologic, morphologic, or phenotypic alteration in the cell. The term "functional fragments of Homer polypeptide," refers to fragments of a Homer polypeptide that retain a Homer activity, e.g., the ability to interact with cell-surface or intracellular receptors or mediate intracellular calcium mobilization, and the like. Additionally, functional Homer
30 fragments may act as competitive inhibitors of Homer binding, for example, biologically functional fragments, for example, can vary in size from a polypeptide

fragment as small as an epitope capable of binding an antibody molecule to a large polypeptide capable of participating in the characteristic induction or programming of phenotypic changes within a cell.

A functional Homer polypeptide includes a polypeptide as set forth in SEQ ID NO:2 and conservative variations thereof. The terms "conservative variation" and "substantially similar" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. The terms "conservative variation" and "substantially similar" also include the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Also included are other Homer nucleic acid and amino acid sequences, including Homer 1b (SEQ ID NOS:3 and 4); Homer 1c (SEQ ID NOS:5 and 6); Homer 2a (SEQ ID NOS:7 and 8); Homer 2b (SEQ ID NOS:9 and 10); Homer 3 (SEQ ID NOS:11 and 12).

Cell-surface receptors are important intermediaries in intercellular signaling. A "cell-surface receptor" is a protein, usually having at least one binding domain on the outer surface of a cell where specific molecules may bind to, activate, or block the cell surface receptor. Cell surface receptors usually have at least one extracellular domain, a membrane spanning region ("transmembrane") and an intracellular domain. Activation of a cell-surface receptor can lead to changes in the levels of various molecules inside the cell. Several types of cell-surface receptors have been identified in a variety of cell types, including ligand-gated receptors, ligand-gated channels, voltage-activated receptors, voltage-activated channels, ion channels and the like.

One class of cell-surface receptor is excitatory amino acid receptors (EAA receptors) which are the major class of excitatory neurotransmitter receptors in the central nervous system. "EAA receptors" are membrane spanning proteins that

mediate the stimulatory actions of glutamate and possibly other endogenous acidic amino acids. EAA are crucial for fast excitatory neurotransmission and they have been implicated in a variety of diseases including Alzheimer's disease, stroke schizophrenia, head trauma and epilepsy. EAA have also been implicated in the process of aging. In addition, EAA are integral to the processes of long-term potentiation, one of the synaptic mechanisms underlying learning and memory. There are three main subtypes of EAA receptors: (1) the metabotropic or trans ACPD receptors; (2) the ionotropic NMDA receptors; and (3) the non-NMDA receptors, which include the AMPA receptors and kainate receptors.

10 Ionotropic glutamate receptors are generally divided into two classes: the NMDA and non-NMDA receptors. Both classes of receptors are linked to integral cation channels and share some amino acid sequence homology. GluR1-4 are termed AMPA (α -amino -3-hydroxy-5-methylisoxazole-4-propionic acid) receptors because AMPA preferentially activates receptors composed of these subunits, while GluR5-7 are termed kainate receptors as these are preferentially sensitive to kainic acid. Thus, an "AMPA receptor" is a non-NMDA receptor that can be activated by AMPA. AMPA receptors include the GluR1-4 family, which form homo-oligomeric and hetero-oligomeric complexes which display different current-voltage relations and Ca^{2+} permeability. Polypeptides encoded by GluR1-4 nucleic acid sequences can form functional ligand-gated ion channels. An AMPA receptor includes a receptor having a GluR1, GluR2, GluR3 or GluR4 subunit. NMDA receptor subtypes include class NR2B and NR2D, for example.

Metabotropic glutamate receptors are divided into three groups based on amino acid sequence homology, transduction mechanism and binding selectivity: 25 Group I, Group II and Group III. Each Group of receptors contains one or more types of receptors. For example, Group I includes metabotropic glutamate receptors 1 and 5 (mGluR1 and mGluR5), Group II includes metabotropic glutamate receptors 2 and 3 (mGluR2 and mGluR3) and Group III includes metabotropic glutamate receptors 4, 6, 7 and 8 (mGluR4, mGluR6, mGluR7 and mGluR8). Each mGluR type may be found in several subtypes. For example, subtypes of mGluR1 include mGluR1a, mGluR1b and mGluR1c. 30

Group I metabotropic glutamate receptors represent a family of seven membrane spanning proteins that couple to G-proteins and activate phospholipase C (Nakanishi, 1994). Members of the family include mGluR1 and mGluR5. Activation of these receptors results in the hydrolysis of memberane phosphatidylinositol
5 bisphosphate to diacylglycerol, which activates protein kinase C. and inositol trisphosphate, which in turn activates the inositol trisphosphate receptor to release intracellular calcium. (Aramori and Nakanishi, 1992; Joly *et al.*, 1995 Kawabata *et al.*, 1998)

Activation of a glutamate receptor on the cell surface results in a cellular
10 response. A "cellular response" is an event or sequence of events that singly or together are a direct or indirect response by a cell to activation of a cell surface receptor. A "cellular response" is also the blockade or activation of selective and non-selective cation channels and potentiation or inhibition of other cell-surface receptor responses. In addition, a "cellular response" may be the activation of an
15 intracellular signaling pathway, including the activation of all steps or any one step in an intracellular signaling pathway.

An "intracellular signaling pathway" is a sequence of events that transduces information about an extracellular event into a signal to intracellular receptors or effector molecules such as enzymes. One type of intracellular signaling pathway is a
20 second messenger signaling pathway. It may begin with the activation of receptors on the cell surface, which activation evokes changes in the level of specific, diffusible molecules inside the cell. The regulated production of these molecules serves to signal events to the intracellular receptors and is therefore termed a second messenger signaling pathway. Major second messenger pathways include the adenylate cyclase
25 pathway, which regulates levels of cyclic AMP, the phosphoinositide pathway, which regulates intracellular calcium, guanylate cyclase, which regulates levels of cGMP, and the nitric oxide pathway, which regulates nitric oxide.

A cellular response mediated by cell surface receptors can also include calcium mobilization. A compound can modulate cellular responses mediated by cell
30 surface receptors by inhibiting or potentiating the release of calcium from intracellular stores. A compound increases calcium mobilization by increasing the release of

calcium from intracellular stores. A compound decreases calcium mobilization by inhibiting of the release of calcium from intracellular stores.

Cell-surface receptors are known to mediate cellular responses. Methods for demonstrating cellular responses are well known in the art (*e.g.* electrophysiological and biochemical methods). (See Examples section for additional methodology). A method is provided for identifying a compound that modulates a cellular response mediated by a cell-surface receptor. The method includes incubating the compound and a cell expressing a cell-surface receptor and a Homer protein under conditions sufficient to permit the compound to interact with the cell. The cell may be any cell of interest, including but not limited to neuronal cells, glial cells, cardiac cells, bronchial cells, uterine cells, testicular cells, liver cells, renal cells, intestinal cells, cells from the thymus and spleen, placental cells, endothelial cells, endocrine cells including thyroid, parathyroid, pituitary and the like, smooth muscle cells and skeletal muscle cells. The cell is exposed to a cell-surface receptor ligand. A "cell surface receptor ligand" is a compound that binds to the binding site of the cell-surface receptor thereby initiating a sequence of events that singly or together embrace a "cellular response". The effect of the compound on the cellular response is determined, either directly or indirectly, and a cellular response is then compared with a cellular response of a control cell. A suitable control includes, but is not limited to, a cellular response of a cell not contacted with the compound. The term "incubating" includes conditions which allow contact between the test compound and the cell of interest. "Contacting" may include in solution or in solid phase.

Compounds which modulate a cellular response can include peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds and biological agents, for example. Antibodies, neurotropic agents, anti-epileptic compounds and combinatorial compound libraries can also be tested using the method of the invention. One class of organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 Daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional

chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups.

The test agent may also be a combinatorial library for screening a plurality of
5 compounds. Compounds such as peptides identified in the method of the invention can be further cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the isolation of a specific DNA sequence. Molecular techniques for DNA analysis (Landegren *et al.*, *Science* 242:229-237, 1988) and cloning have been reviewed (Sambrook *et al.*, Molecular Cloning: a
10 Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, NY, 1998, herein incorporated by reference).

Candidate compounds are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds
15 and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to
20 produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs. Candidate agents are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or
25 combinations thereof.

A variety of other agents may be included in the screening assay. These include agents like salts, neutral proteins, *e.g.*, albumin, detergents, *etc.* that are used to facilitate optimal protein-protein binding and/or reduce nonspecific or background interactions. Reagents that improve the efficiency of the assay, such as protease
30 inhibitors, nuclease inhibitors, antimicrobial agents and the like may be used. The mixture of components are added in any order that provides for the requisite binding.

Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 10 h will be sufficient.

5 In another embodiment, a method is provided for identifying a compound that modulates a cellular response mediated by an intracellular receptor. An "intracellular receptor" is a protein that binds particular intracellular molecules. Intracellular receptors include ryanodine receptors and inositol trisphosphate receptors, for example, an "inositol trisphosphate receptor" is a receptor that binds the compound
10 inositol 1,4,5 trisphosphate, which is an important intracellular second messenger. Inositol 1,4,5 trisphosphate is released from phosphatidyl inositol bisphosphate by the action of a specific phospholipase C enzyme (PLC) and binds to and activates a calcium channel in the endoplasmic reticulum (ER).

 A compound can modulate a cellular response mediated by an intracellular
15 receptor by inhibiting or potentiating the release of calcium from intracellular stores, for example, a compound increases calcium mobilization by increasing the release of calcium from intracellular stores. A compound decreases calcium mobilization by inhibiting of the release of calcium from intracellular stores.

 The method of the invention includes incubating the compound and a cell
20 expressing an intracellular receptor and a Homer protein under conditions sufficient to permit the compound to interact with the cell, exposing the cell to conditions that activate said intracellular receptor, and comparing a cellular response in a cell incubated with said compound with the response of a cell not incubated with said compound. Methods for determining cellular responses mediated by intracellular
25 signals are well known to one of skill in the art (*e.g.*, biochemical assays) and provided in the Examples as well.

 A method is also provided for identifying a compound that modulates receptor-activated calcium mobilization. The term "calcium mobilization" means a change in the amount or concentration of free calcium (Ca^{+2}) sequestered in the
30 endoplasmic reticulum, sarcoplasmic reticulum or mitochondria of a cell. The

method includes incubating the compound and a cell expressing a Homer protein under conditions sufficient to permit the compound to interact with the cell and exposing the cell to conditions sufficient to activate calcium mobilization. Then, the cellular response of the cell exposed to the compound is compared to the cellular response of a cell not exposed to the compound. A difference in a cellular response is indicative of a compound that modulates receptor-activated calcium mobilization in a cell.

In another embodiment of the invention, a method is provided for modulating receptor-mediated calcium mobilization in a cell including exposing a cell to a compound in a sufficient amount to modulate the calcium mobilization that normally occurs when a cell is exposed to an amount of ligand sufficient to activate an intracellular signaling pathway. Those of skill in the art will understand that "the calcium mobilization that normally occurs" depends on the cell type and on the ligand activating the intracellular pathway (Berridge, 1997 *supra*; Berridge, 1998 *supra*; Bootman, 1997 *supra*). Methods of measuring free calcium flux are well known in the art (*e.g.*, imaging methodology using calcium-sensitive dyes such as fura-2 and the like).

A ligand which activates the intracellular signaling pathway may be an agonist or antagonist of metabotropic glutamate receptors. The terms "agonist" and "antagonist" are meant to include compounds that bind to the receptor and, respectively, activate or block activation of the receptor. Known agonists of metabotropic glutamate receptors include glutamate, quisqualate, Ibotenate, homocysteine sulfinate and the neurotoxin β -N-methylamino-L-alanine. Antagonists of metabotropic glutamate receptors include MCPG. Known agonists of the NMDA type glutamate receptor include glutamate and NMDA and known antagonists include MK-801 and APV.

Another embodiment of the invention includes a method of identifying a compound that inhibits Homer protein activity. The method relies on functional properties of the Homer EVH1 and coiled-coil binding domains that can be used to establish high-throughput screens for molecules that influence these and other functional properties of Homer family members. Homer protein activity may be

blocked, partially or completely, by interfering with a protein or other molecule in the intracellular signaling pathway through which Homer proteins act. For example, Homer activity can be modulated, for example, by modulating Homer protein expression, by modifying the activity of the Homer EVH1 domain, by modification of the activity of the Homer CC domain, by modification of Homer crosslinking activity, and the like. Homer activity can also be modulated with by interfering with the expression or activity of Homer Interacting Protein I42, Homer Interacting Protein I30, NR2D, ACK-2, Shank proteins, ryanodine, inositol trisphosphate, and hInaD, and the like

Homer proteins function as a regulated adapter network that cross-links interacting proteins. Cross-linking is determined by the binding properties of the Homer EVH1 domain, which recognize a unique proline-rich ligand with a core sequence consensus of PXXF. This Homer ligand is present in all identified proteins that naturally associate with Homer, and the ability of Homer proteins to bind can be disrupted by single amino acid changes in this motif. Cross-linking activity of Homer proteins has demonstrated effects on glutamate receptor signaling and this action is due to the formation of signaling complexes that link cell-surface receptors with intracellular receptors. Cross-linking by Homer proteins may also have consequences on receptor trafficking or other cellular functions of the interacting proteins.

Development of agents that modulate activity of the Homer EVH1 domain is furthered by knowledge of the crystal structure of Homer protein. The method includes designing inhibitors of Homer protein that form non-covalent bonds with amino acids in the Homer binding sites based upon the crystal structure co-ordinates of Homer protein binding domain; synthesizing the inhibitor; and determining whether the inhibitor inhibits the activity of Homer protein.

The "Homer protein binding domain" is a conserved sequence of amino acids in the amino-terminal region of the that interacts with other proteins. All Homer proteins possess a conserved region of about 175 amino acids at their amino-termini. The 110 terminal amino acids in this region interact with the carboxy-termini of other proteins, for example metabotropic glutamate receptors, inositol trisphosphate receptors, Shank, and the like. The carboxy-termini region of the proteins to which

the Homer protein binding domain may bind usually contains an amino acid sequence that contains a high number of proline residues.

One aspect of the invention resides in the obtaining of crystals of Homer protein of sufficient quality to determine the three dimensional (tertiary) structure of the protein by X-ray diffraction methods. The knowledge obtained concerning Homer proteins may be used in the determination of the three dimensional structure of the binding domain of Homer proteins. The binding domain can also be predicted by various computer models. Upon discovering the three-dimensional protein structure of the binding domain, small molecules which mimic the functional binding of Homer protein to its ligands can be designed and synthesized. This is the method of "rational" drug design. Another approach to "rational" drug design is based on a lead compound that is discovered using high throughput screens; the lead compound is further modified based on a crystal structure of the binding regions of the molecule in question. Accordingly, another aspect of the invention is to provide material which is a starting material in the rational design of drugs which mimic or prevents the action of Homer proteins.

The term "crystal structure coordinates" refers to mathematical coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of X-rays by the atoms (scattering centers) of a Homer protein molecule in crystal form. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are used to establish the positions of the individual atoms within the unit cell of the crystal. The crystal structure coordinates of the Homer protein binding domain are obtained from a Homer protein crystal having orthorhombic space group symmetry $P2_12_12_1$ with $a = 33.79$, $b = 51.40$, and $c = 66.30$ Angstroms. The coordinates of the Homer protein binding domain can also be obtained by means of computational analysis.

The term "selenomethionine substitution" refers to the method of producing a chemically modified form of the crystal of Homer. The Homer protein is expressed by bacterial in media that is depleted in methionine and supplemented in selenomethionine. Selenium is thereby incorporated into the crystal in place of methionine sulfurs. The location(s) of selenium are determined by X-ray diffraction

analysis of the crystal. This information is used to generate the phase information used to construct three-dimensional structure of the protein.

The term "heavy atom derivatization" refers to the method of producing a chemically modified form of the crystal of Homer. A crystal is soaked in a solution containing heavy metal atom salts or organometallic compounds, which can diffuse through the crystal and bind to the surface of the protein. The location(s) of the bound heavy metal atom(s) are determined by X-ray diffraction analysis of the soaked crystal. This information is used to generate the phase information used to construct three-dimensional structure of the protein.

Those of skill in the art understand that a set of structure coordinates determined by X-ray crystallography is not without standard error.

The term "unit cell" refers to the basic parallelepiped shaped block. The entire volume of a crystal may be constructed by regular assembly of such blocks.

The term "space group" refers to the arrangement of symmetry elements of a crystal.

The term "molecular replacement" refers to a method that involves generating a preliminary model of an Homer crystal whose structure coordinates are not known, by orienting and positioning a molecule whose structure coordinates are known. Phases are then calculated from this model and combined with observed amplitudes to give an approximate Fourier synthesis of the structure whose coordinates are known.

The crystal structure coordinates of Homer protein may be used to design compounds that bind to the protein and alter its physical or physiological properties in a variety of ways. The structure coordinates of the protein may also be used to computationally screen small molecule data bases for compounds that bind to the protein. The structure coordinates of Homer mutants (e.g., missense mutations, deletion mutations, and the like, obtained by site-directed mutagenesis, by exposure to mutagenic agents, through selection of naturally occurring mutants, *etc.*) may also facilitate the identification of related proteins, thereby further leading to novel therapeutic modes for treating or preventing Homer-mediated conditions. A potential

inhibitor is designed to form hydrogen bonds with tryptophan²⁴, phenylalanine⁷⁴, threonine⁶⁶, threonine⁶⁸, glutamine⁷⁶, alanine⁷⁸, threonine⁷⁰, and valine⁸⁵ of the Homer binding domain.

5 A method is also provided for identifying a compound that affects the formation of cell surface receptors into clusters. The method includes incubating the compound and a cell expressing a Homer protein and a Homer Interacting protein, such as a Shank protein, a Homer Interacting Protein, and the like, under conditions sufficient to allow the compound to interact with the cell, determining the effect of the compound on the formation of cell-surface receptors into clusters, and comparing the
10 formation of cell-surface receptors into clusters in cells contacted with the compound with the formation of cell surface receptors into clusters in cells not contacted with the compound.

Shank proteins are a novel family of proteins found at the postsynaptic density (PSD) and which are capable of binding to other proteins. Shank proteins contain
15 multiple protein interaction domains, including ankyrin repeats, SH3 domain, PDZ domain, at least one proline rich domain and at least one SAM domain. The PDZ domain of Shank mediates binding to the carboxy-terminus of guanylate kinase associated protein (GKAP), and this interaction is important in neuronal cells for the synaptic localization of Shank proteins. Shank proteins also interact with Homer
20 proteins and therefore Shank and Homer may serve as a protein bridge that links specific proteins that bind to Homer and specific proteins that bind to Shank. Exemplary Shank proteins include Shank 1a, Shank 1b and Shank 3, and cortactin binding protein, and the like.

A compound can affect the formation of cell-surface receptors into clusters by
25 either stimulating the formation of cell-surface receptors into clusters or by inhibiting the recruitment of cell-surface receptors into clusters. When the effect is "inhibition", cell-surface clustering is decreased as compared with the level in the absence of the test compound. When the effect is "stimulation", cell-surface clustering is increased as compared to a control in the absence of the test compound.

A method is further provided for treating a subject with a disorder associated with metabotropic receptors or ion channel receptors comprising administering to the subject a therapeutically effective amount of a compound that modulates Homer protein activity. In yet another embodiment, a method is provided for treating a subject with a disorder associated with Homer protein activity, comprising administering to the subject a therapeutically effective amount of a compound that modulates Homer protein activity.

Essentially, any disorder that is etiologically linked to a glutamate receptor, an inositol trisphosphate receptor, a ryanodine receptor, a Shank protein, I42 (or other Homer interacting proteins) or to a Homer protein could be considered susceptible to treatment with an agent that modulates Homer protein activity. The disorder may be a neuronal cell disorder. Examples of neuronal cell disorders include but are not limited to Alzheimer's disease, Parkinson's disease, stroke, epilepsy, neurodegenerative disease, Huntington's disease, and brain or spinal cord injury/damage, including ischemic injury. The disorder may also be a disorder of a cardiac disorder, a disorder of musculature, a renal disorder, a uterine disorder or a disorder of bronchial tissue. The disorder may be epilepsy, glutamate toxicity, a disorder of memory, a disorder of learning or a disorder of brain development.

Detection of altered (decreased or increased) levels of "Homer protein activity" can be accomplished by hybridization of nucleic acids isolated from a cell of interest with a Homer polynucleotide of the invention. Analysis, such as Northern Blot analysis, are utilized to quantitate expression of Homer, such as to measure Homer transcripts. Other standard nucleic acid detection techniques will be known to those of skill in the art. Detection of altered levels of Homer can also be accomplished using assays designed to detect Homer polypeptide. For example, antibodies or peptides that specifically bind a Homer polypeptide can be utilized. Analyses, such as radioimmune assay or immunohistochemistry, are then used to measure Homer, such as to measure protein concentration qualitatively or quantitatively.

Treatment can include modulation of Homer activity by administration of a therapeutically effective amount of a compound that modulates Homer or Homer protein activity. The term "modulate" envisions the suppression of Homer activity or

expression when Homer is overexpressed or has an increased activity as compared to a control. The term "modulate" also includes the augmentation of the expression of Homer when it is underexpressed or has a decreased activity as compared to a control. The term "compound" as used herein describes any molecule, *e.g.*, protein, nucleic acid, or pharmaceutical, with the capability of altering the expression of Homer
5 polynucleotide or activity of Homer polypeptide. Treatment may inhibit the interaction of the EVH1 domain of Homer with its target protein, may increase the avidity of this interaction by means of allosteric effects, may block the binding activity of the coiled-coil domain of Homer or influence other functional properties of
10 Homer proteins.

Candidate agents include nucleic acids encoding a Homer, or that interfere with expression of Homer, such as an antisense nucleic acid, ribozymes, and the like. Candidate agents also encompass numerous chemical classes wherein the agent modulates Homer expression or activity.

15 Where a disorder is associated with the increased expression of Homer, nucleic acid sequences that interfere with the expression of Homer can be used. In this manner, the coupling of cell-surface and intracellular receptors can be inhibited. This approach also utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents to block transcription or translation of Homer mRNA, either by masking that
20 mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme in disorders associated with increased Homer. Alternatively, a dominant negative form of Homer polypeptide could be administered.

When Homer is overexpressed, candidate agents include antisense nucleic acid sequences. Antisense nucleic acids are DNA or RNA molecules that are
25 complementary to at least a portion of a specific mRNA molecule (Weintraub, 1990, *Scientific American*, 262:40). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are
30 preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target cell. The use of antisense methods

to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, 1988, *Anal.Biochem.*, 172:289).

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix.

- 5 Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, *et al.*, 1991, *Antisense Res. and Dev.*, 1(3):227; Helene, C., 1991, *Anticancer Drug Design*, 6(6):569).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases.

- 10 Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988, *J.Amer.Med. Assn.*, 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

- 15 There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, 1988, *Nature*, 334:585) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will
20 occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

- When a disorder is associated with the decreased expression of Homer, nucleic
25 acid sequences that encode Homer can be used. An agent which modulates Homer expression includes a polynucleotide encoding a polypeptide of SEQ ID NO:2, 4, 6, 8, 10 or 12, or a conservative variant thereof. Alternatively, an agent of use with the subject invention includes agents that increase the expression of a polynucleotide encoding Homer or an agent that increases the activity of Homer polypeptide.

In another embodiment of the invention, there is provided a transgenic non-human animal having a transgene that expresses Homer 1a chromosomally integrated into the germ cells of the animal. Animals are referred to as "transgenic" when such animal has had a heterologous DNA sequence, or one or more additional DNA sequences normally endogenous to the animal (collectively referred to herein as "transgenes") chromosomally integrated into the germ cells of the animal. The transgenic animal (including its progeny) will also have the transgene fortuitously integrated into the chromosomes of somatic cells.

Various methods to make the transgenic animals of the subject invention can be employed. Generally speaking, three such methods may be employed. In one such method, an embryo at the pronuclear stage (a "one cell embryo") is harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into both the germ cells and somatic cells of the resulting mature animal. In another such method, embryonic stem cells are isolated and the transgene incorporated therein by electroporation, plasmid transfection or microinjection, followed by reintroduction of the stem cells into the embryo where they colonize and contribute to the germ line. Methods for microinjection of mammalian species is described in United States Patent No. 4,873,191. In yet another such method, embryonic cells are infected with a retrovirus containing the transgene whereby the germ cells of the embryo have the transgene chromosomally integrated therein. When the animals to be made transgenic are avian, because avian fertilized ova generally go through cell division for the first twenty h in the oviduct, microinjection into the pronucleus of the fertilized egg is problematic due to the inaccessibility of the pronucleus. Therefore, of the methods to make transgenic animals described generally above, retrovirus infection is preferred for avian species, for example as described in U.S. Patent No. 5,162,215. If microinjection is to be used with avian species, however, a recently published procedure by Love *et al.*, (Biotechnology, 12, Jan 1994) can be utilized whereby the embryo is obtained from a sacrificed hen approximately two and one-half h after the laying of the previous laid egg, the transgene is microinjected into the cytoplasm of the germinal disc and the embryo is cultured in a host shell until maturity. When the animals to be made transgenic are bovine or porcine, microinjection can be hampered

by the opacity of the ova thereby making the nuclei difficult to identify by traditional differential interference-contrast microscopy. To overcome this problem, the ova can first be centrifuged to segregate the pronuclei for better visualization.

The "non-human animals" of the invention are murine typically (*e.g.*, mouse).
5 The "transgenic non-human animals" of the invention are produced by introducing "transgenes" into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for microinjection. The use of zygotes as a target for gene
10 transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster *et al.*, *Proc. Natl. Acad. Sci. USA* 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder
15 since 50% of the germ cells will harbor the transgene.

The term "transgenic" is used to describe an animal which includes exogenous genetic material within all of its cells. A "transgenic" animal can be produced by cross-breeding two chimeric animals which include exogenous genetic material within cells used in reproduction. Twenty-five percent of the resulting offspring will
20 be transgenic i.e., animals which include the exogenous genetic material within all of their cells in both alleles. 50% of the resulting animals will include the exogenous genetic material within one allele and 25% will include no exogenous genetic material.

In the microinjection method useful in the practice of the subject invention,
25 the transgene is digested and purified free from any vector DNA *e.g.* by gel electrophoresis. It is preferred that the transgene include an operatively associated promoter which interacts with cellular proteins involved in transcription, ultimately resulting in constitutive expression. Promoters useful in this regard include those from cytomegalovirus (CMV), Moloney leukemia virus (MLV), and herpes virus, as
30 well as those from the genes encoding metallothionin, skeletal actin, P-enolpyruvate carboxylase (PEPCK), phosphoglycerate (PGK), DHFR, and thymidine kinase.

Promoters for viral long terminal repeats (LTRs) such as Rous Sarcoma Virus can also be employed. Constructs useful in plasmid transfection of embryonic stem cells will employ additional regulatory elements well known in the art such as enhancer elements to stimulate transcription, splice acceptors, termination and polyadenylation signals, and ribosome binding sites to permit translation.

Retroviral infection can also be used to introduce transgene into a non-human animal, as described above. The developing non-human embryo can be cultured in vitro to the blastocyst stage. During this time, the blastomeres can be targets for retro viral infection (Jaenich, R., *Proc. Natl. Acad. Sci USA* 73:1260-1264, 1976). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan, *et al.* (1986) in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retro virus carrying the transgene (Jahner, *et al.*, *Proc. Natl. Acad. Sci. USA* 82:6927-6931, 1985; Van der Putten, *et al.*, *Proc. Natl. Acad. Sci USA* 82:6148-6152, 1985). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, *et al.*, *EMBO J.* 6:383-388, 1987). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (D. Jahner *et al.*, *Nature* 298:623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic nonhuman animal. Further, the founder may contain various retro viral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (D. Jahner *et al.*, *supra*).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured in vitro and fused with embryos (M. J. Evans *et al.* *Nature* 292:154-156, 1981; M.O. Bradley *et al.*, *Nature* 309: 255-258, 1984; Gossler, *et al.*, *Proc. Natl. Acad. Sci USA* 83: 9065-9069, 1986; and Robertson *et al.*, *Nature* 322:445-448, 1986). Transgenes can be efficiently

introduced into the ES cells by DNA transfection or by retro virus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a nonhuman animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. (For review see Jaenisch, R.,
5 *Science* 240: 1468-1474, 1988).

"Transformed" means a cell into which (or into an ancestor of which) has been introduced, by means of recombinant nucleic acid techniques, a heterologous nucleic acid molecule. "Heterologous" refers to a nucleic acid sequence that either originates from another species or is modified from either its original form or the form primarily
10 expressed in the cell.

"Transgene" means any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism (i.e., either stably integrated or as a stable extrachromosomal element) which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the
15 transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. Included within this definition is a transgene created by the providing of an RNA sequence which is transcribed into DNA and then incorporated into the genome. The transgenes of the invention include DNA sequences which encode Homer protein-sense and antisense polynucleotides, which may be expressed in a
20 transgenic non-human animal. The term "transgenic" as used herein additionally includes any organism whose genome has been altered by in vitro manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. As used herein, the term "transgenic" includes any transgenic technology familiar to those in the art which can produce an organism carrying an
25 introduced transgene or one in which an endogenous gene has been rendered non-functional or "knocked out".

Antibodies of the invention may bind to Homer proteins or Homer interacting proteins provided by the invention to prevent normal interactions of the Homer proteins and Homer Interacting proteins. Binding of antibodies to Homer proteins or
30 Homer Interacting Proteins can interfere with cell-signaling by interfering with an intracellular signaling pathway. Binding of antibodies can interfere with Homer

protein binding to extracellular receptors, *e.g.*, to NMDA receptors, to metabotropic receptors, and the like. Binding of antibodies can interfere with Homer protein binding to intracellular receptors, *e.g.*, inositol trisphosphate receptors, and the like. Furthermore, binding to Homer proteins or to Homer Interacting Proteins can interfere
5 with cell-surface receptor clustering mediated by Homer family proteins.

The antibodies of the invention can be used in any subject in which it is desirable to administer in vitro or in vivo immunodiagnosis or immunotherapy. The antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the
10 antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the antibodies of the invention
15 can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

The term "antibody" as used in this invention includes intact molecules as well
20 as fragments thereof, such as Fab, F(ab')₂, and Fv which are capable of binding to an epitopic determinant present in an invention polypeptide. Such antibody fragments retain some ability to selectively bind with its antigen or receptor.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1988), incorporated herein by reference). Monoclonal antibodies are made
25 from antigen containing fragments of the protein by methods well known to those skilled in the art (Kohler, *et al.*, *Nature*, 256:495, 1975).

Antibodies which bind to an invention polypeptide of the invention can be prepared using an intact polypeptide or fragments containing small peptides of
30 interest as the immunizing antigen. For example, it may be desirable to produce

antibodies that specifically bind to the N- or C-terminal domains of an invention polypeptide. The polypeptide or peptide used to immunize an animal is derived from translated cDNA or chemically synthesized and can be conjugated to a carrier protein, if desired. Commonly used carrier proteins which may be chemically coupled to the immunizing peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), tetanus toxoid, and the like.

Invention polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See, for example, Coligan, *et al.*, Unit 9, Current Protocols in Immunology, Wiley Interscience, 1994, incorporated by reference).

The antibodies of the invention can be bound to many different carriers and used to detect the presence of an antigen comprising the polypeptides of the invention. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody, or will be able to ascertain such, using routine experimentation.

Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be

specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, puridoxal, and fluorescein, which can react with specific antihapten antibodies.

5 In using the monoclonal and polyclonal antibodies of the invention for the in vivo detection of antigen, e.g., Homer, the detectably labeled antibody is given a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled antibody is administered in sufficient quantity to enable detection of the site having the antigen comprising a polypeptide of the invention for which the antibodies are specific.

10 The concentration of detectably labeled antibody which is administered should be sufficient such that the binding to those cells having the polypeptide is detectable compared to the background. Further, it is desirable that the detectably labeled antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

15 As a rule, the dosage of detectably labeled antibody for in vivo treatment or diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. Such dosages may vary, for example, depending on whether multiple injections are given, antigenic burden, and other factors known to those of skill in the art.

20 The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLES

25 Homer 1a is an IEG and is the original member of a family of proteins that function together as a regulated adapter system that is hypothesized to control the coupling of membrane receptors to intracellular pools of releasable calcium. Homer proteins function at excitatory synapses to couple membrane group 1 metabotropic glutamate receptors (mGluR) to endoplasmic reticulum-associated inositol

trisphosphate receptors (IP3R) (Brakeman *et al.*, 1997; Tu *et al.*, 1998; Xiao *et al.*, 1998). Current studies suggest a broader role for Homer proteins in calcium signaling and receptor trafficking. The Shank family of proteins was identified based on their association with Homer (Naisbitt *et al.*, 1999; Tu *et al.*, 1999). Shank, together with Homer, appears to be part of both the NMDA and group 1 mGluR signaling complexes. By virtue of its interaction with Shank, Homer provides a mechanism to couple NMDA Ca²⁺ influx to intracellular Ca²⁺-induced Ca²⁺ release pools. The inventors have identified additional Homer-interacting proteins that provide insight into the role of Homer in trafficking of group 1 mGluR (e.g., SEQ ID NOS:16, 18, 20). Because these Homer-dependent cellular processes are regulated by the IEG form of Homer (Homer 1a), mechanisms by which Homer proteins can modulate Ca²⁺ dynamics of mGluR and NMDA receptors, as well as regulate receptor trafficking are defined.

Homer family proteins possess an N-terminal EVH1 domain that mediates interactions with mGluRs, IP3R, Shank and other novel proteins. The EVH1 domain has been determined to bind the proline rich motif PPXXFR (Tu *et al.*, 1998). The present invention provides the crystal structure of the Homer EVH1 domain. In complementary studies, genetic approaches were used to identify critical residues in both the EVH1 domain and the ligand that modulate the affinity of the Homer-mGluR (and other Homer-interacting proteins) interaction. This information is essential to an understanding of the integrative cellular actions of Homer proteins. Together, these studies define the molecular basis of specificity of EVH1 interaction with its ligands, and provide insight into how the EVH1 interaction is regulated.

This patent application includes a description of several Homer-interacting proteins that are part of the signaling network that is controlled by Homer (e.g., SEQ ID NOS: 16, 18, 20). Yeast two-hybrid screens and searches of NCBI protein data bases identified a set of known and novel candidate interacting proteins for Homer include the ryanodine receptor, NMDA receptor subunit NR2D, human InaD and novel interacting proteins termed I42 and I30. As described below, current data indicate that agents can be developed that specifically modulate the crosslinking activity of Homer for these various receptors and thereby provide novel therapeutics

that regulate the output of these receptors on cellular function.

Homer acts in several ways to regulate cellular function. Homer and Homer-related proteins function as an adapter system to couple membrane receptors to intracellular pools of releasable Ca. This "signaling" function of Homer is documented in Xiao *et al* (1998), Tu *et al* (1998 and 1999) Naisbett *et al.* (1999), as well as by studies of the novel Homer-interacting protein termed I42 (see below). By virtue of its crosslinking activity, Homer proteins play a role in synaptogenesis and spatial targeting/trafficking of GluRs to other postsynaptic structural proteins. This function of Homer is supported by observations in Tu *et al* (1999) and Naisbett *et al* (1999).

10 Initial cloning of Homer; a novel brain Immediate Early Gene (IEG)

Homer was cloned in a differential screen of seizure-stimulated hippocampus. Prior work, in which IEG induction was examined in brain provided a detailed understanding of time course and tissue distribution of the IEG response (Cole *et al.*, 1989; Saffen *et al.*, 1988; Worley *et al.*, 1990), and suggested a paradigm to maximally induce novel IEG mRNAs (Lanahan and Worley, 1998; Worley *et al.*, 1990). Once cloned, *in situ* hybridization was used to screen for IEGs that were regulated in other paradigms that activate neurons including LTP stimulation in the hippocampus (Brakeman *et al.*, 1997) and acute administration of cocaine. In these models, Homer was one of the most highly induced of all of the IEGs (Brakeman *et al.*, 1997). Initial characterization of Homer was challenging in that the mRNA was nearly 7 kb, while the best deduced open reading frame was only 186 aa, and was located near the 5' end of the cDNA (Brakeman *et al.*, 1997). The 3' UTR was over 5 kb. The ORF was confirmed by *in vitro* transcription and translation of the cDNA, and rabbit polyclonal antisera were generated against bacterially expressed fusion proteins. With these antibodies, we were able to demonstrate that the protein was rapidly and transiently induced in the hippocampus following a seizure (Brakeman *et al.*, 1997). This confirmed the deduced ORF and assured us that the cDNA was indeed translated in brain.

30 Homer selectively binds group 1 metabotropic receptors and is enriched at synapses

In an effort to discover the function of Homer, a yeast 2-hybrid technique (Chevray and Nathans, 1992; Fields and Song, 1989) was used to screen a cDNA library prepared from rat hippocampus and cortex. The full length Homer IEG was used as bait. Among ~30 confirmed interacting cDNAs, one encoded the C-terminal 250 aa of mGluR5. We initially confirmed that the proteins bind using a GSTHomer in a pulldown assay with either fragments of mGluR5, or full length mGluR5 expressed in heterologous cells (HEK293 cells) (Brakeman *et al.*, 1997). Homer protein also bound to mGluR1a, but not mGluR2, 3, 4, or 7. This was an interesting clue to the function of Homer since mGluR1 and mGluR5 (termed group 1 metabotropic receptors) couple to phospholipase C and active hydrolysis of phosphoinositides to generate inositol trisphosphate and diacylglycerol (Nakanishi *et al.*, 1994). mGluR1a and 5 also share sequence similarity in their long, cytosolically disposed C-terminus. Other metabotropic glutamate receptors (termed group 2 and 3) inhibit adenylate cyclase activity, and have short C-termini that lack homology to group 1 receptors. We proceeded to test whether Homer and mGluR5 naturally associate in brain and confirmed that these proteins co-immunoprecipitate from detergent extracts of hippocampus (Brakeman *et al.*, 1997). The next major clue was provided by the observation that Homer immunoreactivity was enriched at excitatory synapses (Brakeman *et al.*, 1997). In brain, Homer protein was associated with dendrites and showed a punctate pattern consistent with a localization in spines. The binding properties and cellular distribution of Homer suggested a role at the excitatory synapse.

Homer is a member of a family of closely related proteins that are enriched at the excitatory synapse

A search of the NCBI sequence data base identified several ESTs that showed strong homology to Homer, but were clearly distinct in that they encoded additional C-terminal sequence (Brakeman *et al.*, 1997). Using a combination of screening strategies, a family of 12 cDNAs was identified from rat, mouse, *Drosophila*, and human (Xiao *et al.*, 1998). All of these cDNAs encoded proteins with a similar protein structure and were deduced to be the products of 3 independent mammalian genes (termed Homer 1, 2, 3) and 1 *Drosophila* gene. Like Homer IEG (now termed

Homer 1a), all new family members contain an N-terminal, ~110 amino acid domain that binds mGluR1a/5 ((Xiao *et al.*, 1998). The region of Homer that interacts with mGluR1a/5 is termed an EVH1 domain based on its modest homology (20-25% identity) to domains in a family of proteins that include *Drosophila* Enabled [Gertler, 5 1996], mammalian VASP (Haffner *et al.*, 1995) and the Wiscott-Aldridge protein (WASP) (Ponting and Phillips, 1997; Symons *et al.*, 1996). The EVH1 domains of Homer proteins from *Drosophila*, rodent and human are conserved at a level of 80% identity (Xiao *et al.*, 1998). Other than the IEG Homer 1a, all new forms of Homer encode an additional C-terminal domain with predicted coiled-coil (CC) structure.

10 As the nomenclature suggests, *Homer 1* gene encodes both the IEG form (Homer 1a) and splice forms that encode CC domains termed Homer 1b and 1c. The 1b and 1c splice forms differ in their inclusion of an approximately 10 amino acid sequence located between the EVH1 and CC domains. (Homer family members that encode CC domains are also referred to as CC-Homers to distinguish them from 15 Homer 1a, which lacks a CC domain.) Similarly, *Homer 2* encodes two CC-Homer splice forms termed Homer 2a and 2b, which also differ by a short internal sequence between EVH1 and CC domains. Homer 3 encodes a single form. The CC domains are less conserved than the EVH1 domain (~40% identity between rat Homer 1, 2 and 3) but they are able to specifically bind to themselves and to CC-domains of other 20 Homer family members (Xiao *et al.*, 1998). Homer CC domains do not interact with other representative CC-domain proteins in GST pulldown assays, and a yeast 2-hybrid screen of brain cDNA with the CC-domain of Homer 1 identified multiple copies of Homer 1, Homer 2 and Homer 3, but not other CC domains (Xiao *et al.*, 1998). As evidence that Homer proteins can naturally self-multimerize, we 25 demonstrated that Homer 1b/Homer 3 heteromultimers co-immunoprecipitate from brain (Xiao *et al.*, 1998). These observations indicate that the Homer CC domains mediate specific self-association.

In contrast to Homer 1a, all CC-containing Homer family members are constitutively expressed in brain (Xiao *et al.*, 1998)]. This was confirmed using both 30 Northern blot and in situ hybridization assays which compared expression with Homer 1a in the same material. mRNA and protein expression of Homer 1b/c, Homer

2 and Homer 3 are unchanged in hippocampus following a seizure while Homer 1a mRNA and protein are induced at least 10 fold.

Antibodies were generated that specifically recognize each of the CC-Homers. Antibodies were raised against synthetic C-terminal peptide sequences. Because
5 Homer 1b and 1c possess identical C-termini, the C-terminal antibodies recognize both splice forms. Similarly, C-terminal Homer 2 antibodies recognize both Homer 2a and 2b. Accordingly, when using these antibodies to detect Homer proteins, we refer to the immunoreactivity as Homer 1b/c or Homer 2a/b. We used these antibodies to determine that Homer 1b/c and 3 are enriched in a detergent resistant fraction of the
10 postsynaptic density (PSD) (Xiao *et al.*, 1998). Homer 2a/b is also enriched in synaptic fractions, but is relatively more soluble than Homer 1b/c and Homer 3. Like Homer 1a, each of the CC-Homers co-immunoprecipitates with group 1 mGluRs from brain (Xiao *et al.*, 1998). Immunogold electron microscopy (EM) demonstrated that Homer 1b/c and Homer 3 are ultrastructurally localized at the PSD (Xiao *et al.*, 1998).
15 These observations suggest that CC-Homer proteins function as multivalent adapter complexes that bind mGluRs at postsynaptic sites.

Homer 1a functions as a natural dominant negative protein.

The fact that Homer 1a lacks a CC domain suggested that it may function as a natural dominant negative to disrupt cross-linking of CC-Homers. In this model, the
20 EVH1 domain of Homer 1a can bind and compete for the same target proteins as CC-Homers (such as mGluR5), but because Homer 1a lacks the CC-domain, it cannot self-associate and cannot cross-link. To test the dominant negative hypothesis, we generated a transgenic mouse that constitutively expressed Homer 1a in brain neurons under the control of a modified Thy-1 promoter [Aigner, 1995 #200]. We confirmed
25 transgene expression in hippocampus, cerebellum and cortex in two independent lines (Xiao *et al.*, 1998). The level of transgene expression in the hippocampus was similar to natural Homer 1a expression induced by a seizure. In contract to the natural Homer 1a, however, the transgene was constitutively expressed in the unstimulated mouse. A prediction of the dominant negative hypothesis is that the ability to co-
30 immunoprecipitate mGluR with Homer 1b/c or Homer 3 antibodies should be diminished in the transgenic mouse. As one of the controls for this experiment, we

demonstrated by western blot that levels of expression of mGluR1a, mGluR5 and Homer 1b/c, 2a/b, 3 were unchanged in the transgenic mouse brain. We then performed IP experiments and observed the anticipated result; the co-immunoprecipitation of mGluR5 with CC-Homers from hippocampus was reduced in the transgenic mouse (Xiao *et al.*, 1998). Similar co-immunoprecipitations of mGluR1a with Homer 3 from cerebellum was also reduced. As an additional control, we demonstrated that the ability to co-immunoprecipitate Homer 1b/c with Homer 3 was not altered in the transgenic mouse. This was the predicted result since the association between these proteins is mediated by their CC domains, and this interaction is not altered by the Homer 1a EVH1 domain. These observations support the hypothesis that Homer 1a functions as a natural dominant negative to regulate CC-Homer-dependent cross-linking.

Homer binds a proline rich sequence that is ~50 aa from the C-terminus of group 1 mGluRs.

When we initially characterized the interaction between Homer and mGluR5, we anticipated that Homer might bind the free C-terminus. This surmise was based on the precedent of PDZ proteins such as PSD95 and GRIP, which bind the free C-terminus of NMDAR2 (Kornau, 1995) and AMPA receptors (Dong *et al.*, 1997). Homer was noted to encode a GLGF sequence like the PDZ domain. Additionally, in GST pulldown assays that used brief washes, we noted a modest reduction of binding when the C-terminal 4 or 10 aa were deleted from mGluR5 [Brakeman, 1997 #99]. (In retrospect, this modest reduction of binding may be due to Homer pulldown of Shank which does bind the free C-terminus of mGluR5, but appears to be lower affinity than Homer-mGluR5 binding; see below.) However, with more standard wash conditions, it became clear that the 4 and 10 aa C-terminal deletion mutants of mGluR5 continued to bind avidly to Homer. We continued the deletion strategy until we found that a 50 aa C-terminal deletion of mGluR5 destroyed binding to Homer. By contrast, a 41 aa deletion of mGluR5 retained full binding activity. We noted that the intervening sequence was proline rich and shared sequence similarity with the previously described SH3 ligand sequence [Yu, 1994 #166] We prepared a series of point mutants based on the known structure-function relationship for SH3 ligands. Binding

assays confirmed general characteristics of SH3 ligand binding, but also demonstrated that the Homer binding site is distinct in the positioning of critical amino acids (Tu *et al.*, 1998). A consensus for binding was determined to be PPXXFR, consistent with the observation that mutation of either of the prolines or the phenylalanine, or a change in their relative position, interrupted binding. The arginine in the last position is preferred over other amino acids, but is not essential. Mutations were identically effective in interrupting binding to each of the Homer family members including Homer 1a, 1b/c, 2a/b, 3 and an EVH1 only fragment (110aa) of Homer 1. Thus, we conclude that the interaction with mGluR5 is mediated by the Homer EVH1 domain.

Mutations of mGluR5 were initially tested in the context of a 250aa C-terminal fragment, but were also determined to have an identical effect on binding when placed in the full length mGluR5 protein (Tu *et al.*, 1998). This exquisite sensitivity of Homer binding to changes in single amino acid within the Homer-ligand site has been confirmed in other Homer-interacting proteins including mGluR1a (Tu *et al.*, 1998), Shank (Tu *et al.*, 1999) and I42 (see below). To further confirm that the interaction was mediated by a direct interaction at the Homer-ligand site (as opposed to a secondary allosteric effect on a remote binding site), we prepared synthetic 10 mer peptides with either the wild type, or F-to-R mutation, and demonstrated that the wild type peptide blocked binding of mGluR1a or mGluR5 to each of the Homer family members [(Tu *et al.*, 1998)]. Approximately half of the binding was blocked at a peptide concentration of 3.4 micromolar. By contrast, the F-to-R mutant peptide did not alter binding at concentrations as high as 340 micromolar.

Homer binds the IP3 receptor.

Armed with a consensus sequence that predicted binding to Homer, we searched the NCBI data base for other proteins that might bind Homer. A Homer-ligand site was identified in the IP3R, dynamin III, a human alpha adrenergic receptor and the ryanodine receptor [(Tu *et al.*, 1998)]. Each of these interactions were determined to be consistent with the known topology of the candidate interacting protein, assuming that Homer proteins are cytosolic. We were able to confirm a biochemical interaction of Homer with the IP3R and dynamin III using GST pull down assays. More importantly, we demonstrated that the IP3R co-

immunoprecipitates with each of the Homer 1b/c, 2a/b and 3 from detergent extracts of cerebellum [(Tu *et al.*, 1998)]. Homer appears to be associated with a substantial portion of IP3R in the cerebellum, since a cocktail of the three Homer antibodies is able to specifically (compared to a cocktail of preimmune serums) co-immunoprecipitate ~50% of the total IP3R in detergent extracts (CHAPS).

CC-Homers function to link mGluR5 and IP3R in a signaling complex.

Based on the prior observations, we examined the hypothesis that CC-Homers might cross-link mGluR and IP3R. This notion was appealing in that the IP3R is part of the signaling network that is activated upon glutamate stimulation of mGluR1/5. Signaling complexes had previously been described including; AKAP proteins which function as scaffolds for specific kinases and their substrates [Lester, 1997 #149], and the *Drosophila* protein InaD which couples the membrane light activated channel with its down stream effector enzyme, phospholipase C [Tsunoda, 1997 #147]. Unlike these other examples of signaling complexes, however, Homer would need to form a bridge between receptors in two different membranes. Functional mGluRs are in the plasma membrane while the IP3R is localized primarily to intracellular endoplasmic reticulum (ER). In support of the notion that ER and plasma membranes can come in close apposition in neurons, we noted that Dr. Kristin Harris (Harvard) described the presence of smooth ER (SER, or spine apparatus) in the spines of hippocampal and cerebellar neurons (Tu *et al.*, 1998). Remarkably, the SER forms close appositions with the plasma membrane that were uniquely localized to the lateral margin of the PSD. These sites are precisely where the group 1 mGluRs are localized (Baude *et al.*, 1993; Lujan *et al.*, 1997; Nusser *et al.*, 1994). The IP3R is present in spines of cerebellar Purkinje neurons where it is associated with the spine apparatus (Sato *et al.*, 1990). (Interestingly, in hippocampal neurons, the RYR is present in the spine apparatus while the IP3R appears to be restricted to the dendritic shaft [reviewed in (Narasimhan *et al.*, 1998)]. Homer 1b/c and 3 are also enriched in the cytosol at the lateral margin of the PSD [(Xiao *et al.*, 1998)]. Thus, available anatomic evidence supported the notion that synaptic mGluRs come in close apposition with SER-associated IP3Rs at sites that are enriched for CC-Homers.

As a first test of the hypothesis that CC-Homers cross-link mGluR and IP3R,

we asked whether we could detect a trimolecular complex of mGluR, Homer and IP3R in brain. Indeed, IP3R antibody specifically co-immunoprecipitated Homer and mGluR1a from cerebellum (Narasimhan *et al.*, 1998). Since IP3Rs are not known to directly interact with mGluR1a, this result supported the hypothesis that Homer
5 bridges these proteins to form a trimolecular signaling complex. A further prediction of the "Homer hypothesis" is that Homer 1a should uncouple the putative mGluR-CC-Homer-IP3R complex. To test this, we monitored the effect of Homer 1a expression on glutamate-induced intracellular calcium release. Plasmids expressing Homer 1a or Homer 1b were transfected along with green fluorescent protein (gene gun) and
10 identified Purkinje neurons were stimulated with quisqualate. A patch electrode containing the Ca²⁺ detector Fura-2 was attached to the soma and a holding potential of -60 mV was applied. Tetrodotoxin and picrotoxin were included in the bath to block synaptic input and EDTA/MgCl₂ was included to assure that measured Ca²⁺ increases in the cell were generated from intracellular stores. Under these conditions,
15 quisqualate-induced Ca²⁺ increases are due to mGluR1-evoked release from IP3R pools (Roche *et al.*, accepted). Expression of Homer 1b did not alter the induced Ca²⁺ transient compared to cells transfected with an empty vector. By contrast, neurons transfected with Homer 1a showed a Ca²⁺ transient that was reduced in amplitude and delayed in time to peak (Tu *et al.*, 1998). This result is consistent with the notion
20 that the IP3 generated by mGluR1a activation of phospholipase C is less effective in releasing Ca²⁺ from the IP3R pools in neurons expressing Homer 1a, and is anticipated if Homer 1a disrupts the physical linkage between mGluR1a and IP3R. Released IP3 must diffuse further, thereby resulting in a lower effective concentration of IP3 at the receptor.

25 **CC-Homers alter trafficking of mGluR1a/5 in heterologous cells.**

We initiated studies to examine the effect of Homer on mGluR5 expression. When wild type mGluR5 was expressed in heterologous cells (HEK293, COS or HeLa) the receptor reached the plasma membrane surface where it was diffusely localized. This was also true when mGluR5 was co-expressed with Homer 1a.
30 However, we noted that co-expression of mGluR5 with Homer 1b resulted in intracellular inclusions of mGluR5 (Roche *et al.*, accepted). This effect of Homer 1b

was dependent on the amount of transfected plasmid and was most obvious when equal amounts of Homer 1b and mGluR5 plasmids were co-transfected. There was a trend for higher level expression of mGluR5 when co-transfected with Homer 1b. When ratios of transfected plasmids were titrated so that total mGluR5 expression was the same (comparing expression with or without Homer 1b), a substantial portion of the total mGluR5 was associated with the intracellular pool when co-expressed with Homer 1b. In these cells, relatively less reached the plasma membrane compared to mGluR expressed alone, or co-expressed with Homer 1a. We further noted that at earlier times after transfection of Homer 1b and mGluR5, mGluR5 showed an enrichment in perinuclear organelles with a reticular pattern throughout the cell that resembled the ER. To assess the nature of the CC-Homer-dependent cellular accumulation, we compared the distribution of mGluR5 with the ER specific marker BIP [B (Roche *et al.*, accepted)]. Staining with BIP antibodies revealed extensive ER present in both transfected and untransfected cells and co-localization with mGluR5. We also noted that the perinuclear organelles were not present within non-transfected cells and therefore appeared to be ER-derived structures unique to cells overexpressing mGluR5 and Homer 1b. These observations suggest that Homer 1b, but not Homer 1a, causes mGluR5 to be retained in the ER.

As an additional assay for ER retention, we examined the status of the carbohydrates present on mGluR5 in cells co-expressing Homer 1a or Homer 1b. If Homer 1b caused mGluR5 to be retained within the ER, then mGluR5 should contain immature, high mannose carbohydrates which are sensitive to digestion with the enzyme Endoglycosidase H (Endo H). Alternatively, if mGluR5 had successfully traveled through the ER and cis Golgi, it would possess mature, complex carbohydrates which would be Endo H resistant. Mature carbohydrates would be anticipated if mGluR5 was on the cell surface or if it was sequestered in a post-Golgi intracellular compartment such as endosomes. We determined that mGluR5 is Endo H resistant when expressed alone or with Homer 1a (Xiao *et al.*, 1998). However, when expressed with H1b, mGluR5 is Endo H sensitive, consistent with the hypothesis that expression of H1b leads to the retention of group I mGluR in the ER.

The subcellular localization of the group II metabotropic glutamate receptor

mGluR2 was the same whether expressed alone or with H1b. In addition, we used a series of mGluR5 constructs containing point mutations within the Homer binding site and found that mutations that disrupt mGluR5/Homer interactions *in vitro* also prevented ER retention of mGluR5 co-expressed with H1b [(Takei *et al.*, 1994)]. mGluR5 P1125L, which does not bind to Homer *in vitro* (Tu *et al.*, 1998), was not retained in the ER when co-expressed with H1b. In contrast, mGluR5 S1126F, which does bind Homer *in vitro*, was ER retained when co-expressed with H1b. Other point mutations in adjacent residues were analyzed and the results were consistent with *in vitro* binding studies [summarized in [B (Ikeda *et al.*, 1995)], demonstrating that mGluR5 is retained within the ER by H1b only when its Homer binding site is intact.

While these experiments were performed in heterologous cells, we also noted enrichment of the group I metabotropic receptor mGluR1a in the ER of Purkinje cells [(Kammermeier *et al.*, submitted). Since Purkinje neurons express particularly high levels of CC-Homers [(Xiao *et al.*, 1998)], this suggests Homer proteins may naturally regulate receptor trafficking through the ER. In this model, Homer 1a would be permissive for transfer through the ER Golgi system to insertion into the postsynaptic membrane. The ability of CC-Homers to alter the spatial distribution and metabolism of ER associated proteins may also impact the IP3R. IP3Rs in Purkinje neurons are associated with dense stacks of ER (Satoh *et al.*, 1990) and this stacking morphology has been shown to be regulated by neural activity (Takei *et al.*, 1994). Since a substantial portion of IP3R in cerebellum is associated with CC-Homers, it is possible that the ability of CC-Homer to crosslink interacting proteins on two adjacent membranes plays a regulatory role in ER morphology and function. Experiments in Aims 2 and 3 will examine this hypothesis.

Homer Modulates mGluR coupling to ion channels.

Group 1 mGluRs modulate ionic currents by activating pertussis toxin-sensitive and -insensitive G proteins (Naisbitt *et al.*, 1999). Modulation of Ca²⁺ currents by heterologously expressed group 1 mGluRs in superior cervical ganglion (SCG) neurons proceeds through multiple pathways involving both the α and $\beta\gamma$ subunits of G proteins. We examined the effect of Homer on mGluR coupling to Ca²⁺ and M-type potassium channels in SCG neurons.

CC-Homers, including 1b, 2b and 3 produced a similar reduction of the effect of group 1 mGluRs (Kim *et al.*, 1997; Naisbitt *et al.*, 1999; Naisbitt *et al.*, 1997; Takeuchi *et al.*, 1997). By contrast, Homer 1a or an engineered short form of Homer 2 did not block group 1 mGluR effects, but were able to partially reverse the effect of the CC-Homers.

Homer interacts with Shank suggesting a role synaptogenesis and NMDAR function.

To gain further insight into the physiological function of Homer, we characterized a novel family of proteins that were identified based on their interaction with Homer 1a in a yeast 2-hybrid screen of a brain cDNA library. These Homer-interacting proteins were determined to be identical to the Shank family of PSD proteins that interact with GKAP and the PSD-95 complex (Tu *et al.*, 1999). Shank proteins are specifically enriched at excitatory synapses and co-localize with NMDA receptors in primary neuronal cultures (Naisbitt *et al.*, 1999). Shank proteins appear to be recruited to excitatory synapses by virtue of their interaction with GKAP, a synaptic protein that binds to the guanylate kinase domain of PSD-95 (Kim *et al.*, 1997; Naisbitt *et al.*, 1999; Naisbitt *et al.*, 1997; Takeuchi *et al.*, 1997). In addition to the PDZ domain which binds GKAP, Shank contains domains that mediate self-multimerization and interaction with cortactin (Golshani *et al.*, 1998). Shank also directly interacts with Homer [(Lujan *et al.*, 1997)]. Homer and Shank proteins co-localize at the PSD of CA1 pyramidal neurons [(Tu *et al.*, 1999)], and native Homer-Shank complexes were identified in brain using GST pull down assays of Shank with GKAP [(Otani and Connor, 1998)]. Additionally, Homer and Shank co-immunoprecipitate from brain [(Aniksztejn *et al.*, 1991; Ben-Ari *et al.*, 1992)]. These observations indicate that Shank and Homer naturally associate in brain. Biochemical studies indicate that the Shank-Homer interaction is mediated by the EVH1 domain of Homer which binds to a single Homer-ligand site present in the proline-rich domain of Shank proteins [(Tu *et al.*, 1999)]. A quaternary complex of Homer/Shank/GKAP/PSD-95 is assembled in heterologous cells, with Homer and PSD-95 co-localizing in large clusters [(Berridge, 1998)]. Thus, Shank provides a molecular bridge that links the NMDA receptor complex with Homer and its

associated proteins.

The Homer-Shank interaction also produces clustering of group 1 mGluRs [(Satoh *et al.*, 1990; Villa *et al.*, 1992)]. Clustering molecules have previously been identified for a variety of receptors and ion channels (Selig *et al.*, 1995), but Shank-
5 Homer are the first clustering proteins for group 1 mGluR. It is notable that the mechanism of clustering involves a linkage of mGluRs with the previously defined NMDA receptor scaffold. Thus the Shank-Homer interaction could be relevant to synaptogenesis, by docking mGluRs to a preestablished "core" of NMDA receptors. In support of such a mechanism, functional NMDA receptors appear to precede the
10 emergence of metabotropic receptors in the hippocampus and cerebellum (Xiao *et al.*, 1998). Homer proteins, in association with Shank, could function to localize and cluster the mGluRs in proximity to NMDARs, and may contribute to the perisynaptic localization of group 1 metabotropic receptors (Lujan *et al.*, 1997).

By linking NMDA and mGluR signaling pathways, the Shank-Homer
15 interaction might also contribute to examples of glutamate receptor crosstalk for which physical proximity of molecules may be important, such as activation of phospholipase C (Beneken *et al.*, submitted) or protein kinase C (Aniksztejn *et al.*, 1991; Ben-Ari *et al.*, 1992). Additionally, the Homer/Shank/GKAP/PSD-95 assembly may mediate physical association (and perhaps functional coupling) of the NMDAR
20 with IP3R/RyR and intracellular Ca²⁺ stores. Consistent with such a functional interaction, recent studies indicate that NMDA receptor-dependent increases in spine Ca²⁺ may derive from intracellular stores by a mechanism of Ca²⁺-induced Ca²⁺ release (CICR) [(Emptage *et al.*, 1999) and reviewed by (Svoboda and Mainen, 1999)]. Both IP3R and ryanodine receptor channels possess CICR properties
25 (Berridge, 1998), and are similarly localized in dendrites and spines of specific neuronal types (Satoh *et al.*, 1990; Villa *et al.*, 1992). The physical proximity of glutamate receptors with calcium pools may underlie synergistic effects of mGluRs on NMDA-dependent responses as reported in studies of LTP [(Bashir *et al.*, 1993; Bortolotto *et al.*, 1994) but see also ((Selig *et al.*, 1995)], and is consistent with the
30 reduction of LTP in group 1 mGluR mutant mice [(Prehoda *et al.*, 1999) but see also (Conquet *et al.*, 1994)].

The proposed model for Shank and Homer-dependent clustering requires that Homer be multivalent in order to cross-link Shank/GKAP/PSD95 to IP3R/RYRs and to mGluRs. This is achieved by multimerization of constitutively expressed CC-Homers (Xiao *et al.*, 1998). In this context, the monovalent Homer 1a IEG product appears to
5 function to uncouple proteins that are linked via the constitutively expressed CC-Homer multimers, and thereby dynamically regulate the assembly of this postsynaptic network. Cocaine-induced increases in Homer 1a may thus modulate both mGluR and NMDA Ca²⁺ responses in spines.

Homer EVH1 domain crystal structure.

10 To investigate the structural basis of interactions between EVH1 domains and ligands, we determined the high-resolution crystal structure of the EVH1 domain from rat Homer 1 (). Methods of protein purification and crystallization are described in our manuscript (Niebuhr *et al.*, 1997; Tu *et al.*, 1998). This structure revealed that the EVH1 module is homologous to both the plextrin homology (PH) domain and the
15 phosphotyrosine binding (PTB) domain. (legend next page)

At the same time we were working to solve the structure of Homer 1 EVH1, Dr. Wendel Lim's group (at UCSF) solved the structure of the related EVH1 protein termed Mena (20% identical to Homer EVH1 domain) (Prehoda *et al.*, 1999). Comparison of the Mena and Homer coordinates confirmed that these are related
20 proteins despite the low degree of amino acid identity. The Mena crystal was solved with a 6mer peptide and identified a putative ligand binding surface. Both of our groups determined that co-crystals were not formed with longer synthetic peptides. One issue that concerned us regarding the putative ligand-binding site on Mena was that the affinity of the 6mer used for Mena was 100 fold less than that of a 10 mer
25 (Prehoda *et al.*, 1999). The measured affinity of the 6mer was ~600 micromolar. Additionally, within the EVH1 family, Homer is one of the most divergent members (Prehoda *et al.*, 1999). One important difference between Mena and Homer EVH1 binding, is the orientation of the phenylalanine relative to the polyprolines. The optimal ligand for Mena is FPPPP while the consensus ligand for Homer is PPXXF.
30 This may be important since the F is the single most critical side chain for the interaction when tested with larger peptides for both EVH1 domains (Niebuhr *et al.*,

1997; Tu *et al.*, 1998). In the Mena structure, the F side chain is not placed in a clear hydrophobic pocket (the ring appears to coordinate an arginine) and superposition of the ligand coordinates in Homer EVH1 is even less obviously stabilized.

To examine the predictive power of the Mena co-crystal for the ligand
5 binding activity of Homer EVH1, we tested a series of missense mutations that targeted sites anticipated to contact the prolines of the ligand (PPXXF) sequence. Based on the homology of the EVH1 domain with the PTB domain, we also tested sites on Homer that would be critical if Homer mimicked the peptide binding surface of the PTB domain. This PTB ligand site is remote from the putative Mena EVH1
10 ligand site. Our mutation analysis also tested a series of mutants selected based on the homology between Homer and WASP. Genetic data from patients with Wiscott Aldrich syndrome defined a series of mutations in the EVH1 domain that map to sites that are distinct from both the PTB and the putative Mena ligand sites. Our selection of the mutational substitutions was based on the Homer EVH1 structure. Substituted
15 amino acids were selected to be sufficiently conservative as not to disrupt the primary structure.

A total of 30 missense mutants of the Homer EVH1 domain were expressed in HEK293 cells and assayed for binding to either mGluR1a or Shank3 using GST pulldown assays. Surface-exposed mutations within the region homologous to the
20 peptide binding site of PTB domains had no affect on peptide binding. Similarly, mutations based on the WASP data were also ineffective in disrupting binding. By contrast, certain of the mutants based the Mena ligand site did disrupt Homer EVH1 binding. Despite ambiguities involved with interpreting the effects of any single mutation, the nature and distribution of the effects of site-directed mutations in the
25 Homer EVH1 domain on Homer-ligand interactions strongly implicate the Mena ligand region as mediating natural ligand binding by the Homer EVH1 domain.

One interesting finding from our analysis of mutant Homer EVH1 binding is that certain mutations disrupt binding specifically to mGluR1a, but not to Shank3 (and visa versa). One interpretation of this finding is that there are determinants of
30 binding in addition to the core PPXXF motif. An important implication of this observation is that differences in critical determinants of Homer binding to its various

targets may be exploited to develop pharmaceuticals that can selectively disrupt interactions with a particular target.

I42 Interacts with Homer.

I42 (SEQ ID NOS:17 and 18) encodes a novel protein that was first identified
5 in a Y2H screen of brain cDNA with the Homer EVH1 domain. Current information indicates that I42 functions with Homer at the excitatory synapse. We have generated I42 specific antisera and can demonstrate robust co-immunoprecipitation of I42 with Homer from brain. ImmunoEM analysis demonstrates that I42 is localized to the postsynaptic density. The predicted domain structure of I42 indicates that it shares
10 certain properties with Shank including a N-terminal structural domain (a band 4.1 domain in I42), a single PDZ domain, and a central proline rich domain with a single Homer-ligand site. Additionally, there is a C-terminal type 1 PDZ ligand motif. We have identified a related sequence in the data base (KIAA sequence has several errors with frame shifts) suggesting that I42 may represent a gene family.

15 Current studies indicate a functional interaction of I42, Homer and mGluRs. We have performed a yeast 2-hybrid screen of the I42 PDZ domain and find it binds β -Pix (also termed Cool-1) (Allen *et al.*, 1998). β -Pix is a guanine nucleotide exchange factor (GEF) for Rac1/CDC42. This interaction appears robust using GST pulldown assays and we have recently confirmed the interaction using co-
20 immunoprecipitation assays from brain. Biochemical assays indicate that the PDZ domain of I42 binds its own C-terminus (may be intra or inter molecular). Based on these observations, I42 functions as a scaffold/cytoskeletal regulatory protein that responds to specific signals and may link between mGluR activation and Rac-dependent cytoskeletal remodeling. This biochemical association may play a role in
25 mGluR trafficking or synaptic remodeling. An additional functional consequence of the Homer I42 interaction is indicated by the demonstrated association of β -Pix with p21 activated kinase (Pak) (Tu *et al.*, 1999). Paks are a family of kinase that can signal both locally and more distally to the nucleus. A mutation of Pak3 has recently been linked to mental retardation (Tu *et al.*, 1998), confirming the importance of this
30 regulated kinase to cognitive function. Accordingly, I42 appears to be part of a novel signaling pathway for the mGluRs that may be regulated by Homer proteins.

In preliminary studies, we observe that I42 co-immunoprecipitates with Homer from brain. Antibodies for I42 also co-immunoprecipitates mGluR1 from brain. In parallel studies, we observed the interaction between I42 and β -Pix (). These observations indicate the involvement of Homer in the function of I42/ β -Pix and identify another signaling pathway that can be manipulated by agents that modulate Homer binding function.

ii) Ultrastructural localization of I42/ β -Pix/Pak at synapses: We have performed preliminary immunoEM with I42 Ab and observes that it is associated with the PSD region. The methods and approach are identical to our studies of Shank (Naisbitt *et al.*, 1999). This observation indicates that I42 is enriched at the excitatory synapse together with Homer, Shank and glutamate receptors.

Ryanodine receptor (RYP) and Homer.

The RYP encodes a potential Homer binding site near the N-terminus (Bhat *et al.*, 1999) and using GST pulldown assays we observe that GSTHomer binds to the relevant fragment of RYP1. Importantly, we have demonstrated that the RYP co-immunoprecipitates with Homer from detergent extracts of skeletal muscle. The interaction between RYP and Homer is understood to be consistent with the function of Homer proteins to regulate the coupling of membrane receptors with intracellular calcium pools. Glutamate mediates an inhibitory postsynaptic potential in dopamine neurons of the midbrain and this is mediated by mGluR1 release of intracellular Ca^{2+} from RYP sensitive CICR pools (Bhat *et al.*, 1999). RYP have recently been implicated as an important source of NMDAR-induced calcium rise in the post synaptic spine (Emptage *et al.*, 1999). Since Shank is part of the NMDA receptor signaling complex (Naisbitt *et al.*, 1999) and binds Homer, it is compelling to evaluate the possible interaction between RYP and Homer.

NMDA Receptor Type 2D (NR2D) and Homer.

Independent Y2H screens of adult cortex and cerebellum identified several clones of the NMDA receptor type 2D (NR2D). NR2D has not been as extensively studied as NR2B but is expressed in developing cerebellum and interneurons in the

forebrain (Dunah *et al.*, 1998; Goebel and Poosch, 1999). NMDAR that include the NR2DR have slower channel properties (Cull-Candy *et al.*, 1998; Okabe *et al.*, 1998; Vicini *et al.*, 1998). The C-terminus of NR2D is highly proline rich consistent with our observation that Homer binds a specific proline rich sequence. Thus, in the case of

5 NR2D, Homer proteins form a direct coupling to CICR pools. This direct coupling would contrast with NMDAR that include NR2B which appear to couple to Homer indirectly via PSD95-GKAP-Shank (Naisbitt *et al.*, 1999). In both cases, modification of Homer crosslinking activity will alter the intracellular release of calcium due to glutamate receptor activation. Because of the differences in the binding properties of

10 the EVH1 domain of Homer to its different targets, it is anticipated that agents that specifically disrupt the linkage of NR2B or NR2D can be developed.

Mammalian InaD like molecule interaction with Homer.

We have identified two distinct novel members of a family of proteins with similarity to the recently reported human InaD (Philipp and Flockerzi, 1997) and

15 *Drosophila* Discs Lost [DLT (Bhat *et al.*, 1999)]. These proteins encode 5 and 4 PDZ domains, respectively, and a proline rich region that is shared in all clones that is presumed to mediate interaction with Homer. DLT has been demonstrated to be essential for establishment of epithelial cell polarity and binds to the C-terminus of Neurexin IV [DLT (Bhat *et al.*, 1999). We currently refer to our clones as rat InaD. In

20 current studies, we observe that full length myc-tagged rInaD co-immunoprecipitates with Homer 2 from co-expressing HEK293 cells.

I30 interaction with Homer.

I30 is a novel member of the family of abl binding proteins. Related proteins function as adaptor proteins that regulate cell growth [Ziennicka-Kotula, 1998 #392; Biesova, 1997 #393] and are hypothesized . I30 encodes a SH3 domain and a Homer

25 binding site. Accordingly, Homer is anticipated to link this protein to other Homer-interacting proteins including metabotropic glutamate receptors and IP3R. (See SEQ ID NOS: 15, 16, 19 and 20).

Cdc42-associated tyrosine Kinase-2 (ACK-2) interaction with Homer

ACK-2 is a non-receptor tyrosine kinase that is regulated by the Rho-related GTP-binding protein Cdc42 [Yang, 1999 #391]. ACK-2 is activated by signals that result from cell adhesion, by for example activation of the integrin receptor. One cellular consequence of ACK-2 activation is down stream activation of c-Jun kinase.

- 5 Our observation that ACK-2 interacts with Homer indicates that this signaling pathway can be linked to other membrane receptors by Homer, and identifies another signaling cascade that can be manipulated by agents that alter Homer crosslinking function.

10

EXAMPLE 1

Identification and Sequencing of Homer Family Members

- Low stringency screens of phage cDNA libraries and EST Database searches were performed to identify Homer family members. cDNA libraries were screened using the rat Homer 1a coding region as a probe. Screens of mouse and rat brain
15 cDNA libraries identified two isoforms of Homer-1 (Homer-1b and Homer-1c).

- Searches of EST Databases identified a mouse EST sequence (ID#442801) which is about 73% homologous to a portion of 5' coding region of Homer-I cDNA sequence. Based on the EST used RT-PCR (Forward: 5'-GAC AGC AGA GCC AAC ACC GTG-3'; Reverse: 5'-GTC TGC AGC TCC ATC TCC CAC-3') to
20 amplify the corresponding region from various mouse tissues. The PCR products (~330 bp) consisted of two different sequences, one of which contains an additional insertion of 33 bp. A mixture of these two cDNA fragments were used as probes to screen an adult mouse brain cDNA library. Out of 10⁶ clones screened, five clones hybridized well to the probe. Sequence analysis of these clones indicated that they
25 are five partial cDNA clones representing two isoforms of a Homer-2 gene. These clones are identical to the isoforms amplified by RT-PCR. The 5' region of Homer-2 was cloned using 5'-RACE technique. Total RNA from E14.5 mouse brain was reverse-transcribed using the reverse primer described above. Another gene-specific primer (5'-CAC GGT GTT GGC TCT GCT GTC-3') was used in the amplification of
30 the 5' region of Homer-2. The sequence authenticity of the 5' RACE clones was

further confirmed by sequencing a partial mouse EST clone #441857.

A search of the EST Database allowed the identification of several human EST's corresponding to mouse and rat Homer-1b, Homer-2a and 2b cDNA sequences. RT-PCR was used to clone the human Homer-1b and Homer 2a and 2b coding regions. A 5' degenerate primer (5'-ATG GG(A/G/C) GA(A/G) CA(A/G) CC(T/C/G) AT(T/C) TTC-3') was designed based on an amino-terminal seven residue amino acid sequence (MGEQPIF) that is conserved among human EST clone #HCE003, mouse, rat, and *Drosophila* Homer homologue sequences. The 3' primers (5'-GAG GGT AGC CAG TTC AGC CTC-3') for human Homer-1 and human Homer-2 (5'-GTT GAT CTC ACT GCA TTG TTC-3') were made from the sequences of human EST clones #562862 and #HIBAB15 respectively. Human Homer-1b and Homer-2a and 2b were amplified from new born human frontal cortex. The sequences of human Homer 1b, Homer 2a and Homer 2b were derived from sequencing several PCR clones and EST clones and are shown in SEQ ID NO's:3, 7 and 9.

Human and mouse Homer-3 were identified by searching EST Database, using Homer-1 and Homer-2 sequences. Two full-length human Homer-3 clones were identified (Clone ID #284002 and #38753) and sequenced. Numerous mouse Homer-3 clones were found and one of them (Clone ID #1162828) contains an almost full-length coding region. Also identified were several *Drosophila* EST sequences exhibiting significant homology at the amino acid level to the N-terminal region of Homer family members. The sequence presented in SEQ ID NO:11 is derived from Clone #LD3829.

Expression Constructs Mammalian expression constructs were made by cloning cDNA into Sall and NotI sites of pRK5 (Genentech), so that the cDNA was fused in-frame to an N terminal c-Myc tag. GST-fusion constructs were made by cloning Homer cDNA into the Sall and NotI sites of pGEX4T-2 (Pharmacia). The full-length coding regions of mouse Homer-1b, rat Homer-1c, mouse Homer-2b and human Homer-3 were engineered with Sall and NotI sites at the 5' and 3' ends by PCR using high fidelity DNA polymerase *Pfu* (Stratagene). Various truncations of Homer-1b/c and Homer-2b coding regions were made by PCR with specific Primers containing Sall and NotI sites. All the PCR-based constructs were sequenced to confirm the sequences and in-frame fusion.

The sequence of Homer 1a was used to screen cDNA libraries prepared from rat and mouse brain for related gene products. Homer 1a sequence was also used to search GenBank data bases. Several related rodent and human sequences were identified.

cDNAs that are most closely related to Homer 1a appear to represent alternative splice forms. This inference is based on nucleotide sequence identity of their 5'UTRs and the first 175 amino acids of the open reading frames (ORF). The presumptive novel splice variants, termed Homer 1b and 1c, are completely divergent from Homer 1a after residue 175 of the ORF and they possess entirely distinct 3'UTRs. comparison at the point of sequence divergence indicates that Homer 1a encodes a unique eleven amino acid carboxy terminus of the ORF and about 5 kb 3' UTR region. The unique eleven amino acid carboxy-terminal sequence of Homer 1a does not possess a recognizable motif. In Homer 1b and 1c, an additional 168 and 180 amino acids are present that are predicted to possess coiled-coil (CC) secondary structure (Lupas, *Trends Biochem. Sci* 21:375 (1969)). While the 3'UTR sequence of Homer 1a includes multiple AUUUA repeats which are implicated in destabilizing mRNAs of intermediate early genes (IEG) (Shaw and Kamen, *Cell* 46:659 (1986)), the 3'UTR sequence of Homer 1b and 1c does not include this motif. The only difference between Homer 1b and 1c is the inclusion in Homer 1c of a twelve amino acid sequence insertion at residue 177, between the conserved amino-terminus and the CC domain. Thus, Homer 1b and 1c appear to be formed by a splicing event that

substitutes a relatively long and unique carboxy-terminus of the ORF and shorter 3'UTR sequence that lacks the characteristic IEG motif. Multiple independent isolates of rat and mouse Homer 1b and 1c were identified and sequenced to confirm their natural expression in brain.

5 Further searches identified cDNA sequences that appear to represent two additional Homer genes, termed Homer 2 and Homer 3. The sequences of two splice forms of Homer 2 and one Homer 3 sequence is presented (See Figures section). The predicted size of the protein products and general domain structure are similar to Homer 1b and 1c. Like Homer 1b and 1c, each of the Homer 2 and Homer 3 proteins
10 contain about 120 amino acids at the amino-terminal that is highly similar to the amino-terminal domain of Homer 1a. The degree of amino acid identity in these regions is about 88% between Homer 1 and Homer 2 and about 86% between Homer 1 and Homer 3. Many of the amino acid differences are conservative.

In contrast to the high degree of conservation in amino-terminal region, the
15 carboxy-terminal regions of Homer 2 and 3 are only about 22% identical to Homer 1b, but like Homer 1b and 1c are predicted to possess a CC secondary structure. The CC domains of all Homer family members exhibit significant homology (about 40-45% amino acid similarity) to the CC regions of myosin heavy chain (Strehler *et al.*, *J Mol Biol* 190:291 (1986)), kinesin heavy chain (Yang *et al.*, *Cell* 56:879 (1989)) and
20 dynactin (Gill *et al.*, *J Cell Biol* 115:1639 (1991)). The distinct splice forms of Homer 2, termed Homer 2a and Homer 2b, are differentiated by an eleven amino acid insertion at residue 131 in Homer 2b. Human Homer 1, 2 and 3 are mapped to chromosomes 5, 15 and 19, respectively by the Human Genome Project.

Drosophila Homer possess the basic domain structure of mammalian Homers.
25 The amino-terminus is highly homologous to that of mammalian Homer and the carboxy terminus is predicted to form a CC secondary structure.

EXAMPLE 2

Generation and Characterization of Homer Antisera

Rabbit polyclonal antibodies were generated against synthetic peptides derived from the unique carboxy termini of Homer 1b/c, Homer 2a/b and Homer 3. Synthetic carboxy-terminal peptides of Homer 1, 2 or 3 were conjugated to thyroglobulin with glutaraldehyde and used to immunize rabbits according to a previously published protocol (Martin *et al.*, *Neuron*, **9**:259 1992). Peptide sequences used are contained in Homer-1b and 1c: IFELTEL RDNLAKLLECS; Homer-2a and 2b: GKIDDLHDFRRGLSKLGTDN; and Homer-3: RLFELSELREGLARLAEAA. Detergent (2% SDS) extracts from rat cortex, hippocampus, and cerebellum were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were probed with polyclonal anti-Homer sera. Specificity was tested by incubating the antiserum with 10 µg/ml of relevant peptide at room temperature for 10 min prior to use. Rabbit polyclonal antiserum was also generated against the full length GST-Homer 1a fusion protein, as described previously (Brakeman, *et al.*, *Cell* **87**:227 1997). This antiserum recognizes all Homer 1 isoforms.

Unpurified antibodies were tested for their sensitivity and specificity in detecting heterologously expressed, full length Homer proteins with amino-terminal c-myc tags. Each Homer protein was selectively detected on Western blot by the appropriate Homer antibody in soluble extracts of transfected HEK293 cells. The myc-tagged Homer proteins migrated with an apparent molecular mass of 50 kDa. There was no cross reactivity between antibodies for one Homer form and other family members.

EXAMPLE 3

In Vitro Interaction of Homer Proteins with Cell-Surface mGlu Receptors

To examine the interaction of Homer proteins with mGluR1 and mGluR5, HEK293 cells were transiently transfected (using calcium phosphate) with full length mGluR1α and mGluR5 constructs in pRK5 (Brakeman *et al.*, 1997). Cell lysates were made 24-48 h post-transfection. GST fusion proteins bound to glutathione agarose were prepared of Homer 1a, Homer 1c, Homer 2b, Homer 3 and two amino terminal

fragments of Homer 2 according to the following procedure. GST fusion constructs were prepared by polymerase chain reaction with specific primers that included SalI and NotI sequences and subcloned into pGEX4T-2 vector (Pharmacia Biotech, Uppsala, Sweden). Constructs were confirmed by sequencing. GST-fusion proteins
5 were expressed in BL21 bacterial strains. Bacteria were harvested and lysed in PBS, 1% Triton X100, 2 mM phenylmethylsulfonyl fluoride (PMSF) and pelleted at 13,000 rpm (Sorvall SS-34) at 4°C for 5 m. Proteins were purified by incubating 1 ml bed volume glutathione-sepharose (GST) beads (Sigma USA) with bacterial supernatant at 4°C for 10 m, washing twice with PBS and PBS plus 1% Triton X-100.
10 Protein was eluted with 10 mM glutathione and dialyzed against PBS at 4°C. Protein concentrations were measured by BCA (Pierce, Illinois). Cell lysates of the transfected cells were incubated with equivalent amounts of various Homer-GST fusion proteins at 4 °C for 2 h, washed with PBS and 1% Triton X-100. Proteins were eluted in 2% SDS sample buffer and separated on 8% or 2.5% SDS-PAGE gels and
15 probed with appropriate antibody.

It has been previously demonstrated that the amino-terminal 131 amino acids of Homer 1a is sufficient to bind group I metabotropic glutamate receptors (Brakeman *et al.*, *Nature* 386: 284 (1997)). In view of the high degree of sequence conservation in this region of Homer family members, the possibility that they would also bind
20 group I receptors was examined. GST fusion proteins were prepared of Homer 1a, Homer 1c, Homer 2b Homer 3 and two amino-terminal fragments of Homer 2. The fusion proteins were bound to glutathione agarose and assayed for binding to full length mGluR5 or full length mGluR1a expressed in HEK293 cells. These studies show that mGluR5 bound GST Homer 1a. mGluR5 also bound to all full length
25 Homer constructs and to a Homer 2 amino-terminal fragment of about 141 residues but not to GST alone. The relative binding in the three assays were comparable for each of the three Homer types. A Homer 2 deletion mutant that includes only the amino-terminal 92 residues did not bind mGluR5. Similar binding of Homer proteins to mGluR1 was also observed.

EXAMPLE 4

In Vivo Interaction of Homer Proteins with Cell-Surface mGlu Receptors

To examine if Homer proteins are naturally associated with group I metabotropic receptors in the brain, immunoprecipitation studies were performed.

5 Rat or mouse brain tissues were sonicated (3 x 10 s) in PBS (~200 mg/ml wet weight) containing 1% Triton-X100 with protease inhibitors and centrifuged for 10 m at 15,000g. Three µl of antiserum directed against Homer 1b, Homer 1c, Homer 2a, Homer 2b or Homer 3 was added to 60 µl of tissue extract and incubated for 1-1/2 h at 4°C and then washed three times with PBS/Triton. Preimmune and peptide-

10 blocked antisera were used as negative controls. Binding in tissue samples was analyzed by gel electrophoresis and western blot analysis. Proteins were eluted in 2% SDS loading buffer. mGluR1α monoclonal antibody was obtained from PharMingen (San Diego CA). Rabbit polyclonal mGluR5 antibody was a gift from Dr. Richard Huganir, Johns Hopkins School of Medicine.

15 Homer family members are naturally associated with group I metabotropic receptors in brain. This analysis was performed using cerebellum since all three Homer family members are expressed in this tissue. Detergent extracts of whole adult rat cerebellum were incubated with antibodies to Homer 1b/c, Homer 2a/b or Homer 3 and immunoprecipitates were blotted with a mouse monoclonal antibody to mGluR1α.

20 mGluR1α co-immunoprecipitates with each of the antisera directed against Homer proteins. The predominate band after electrophoreses corresponded to the monomer form of mGluR1α (about 150 kDA) and other bands corresponding to multimers of mGluR1α are also observed.

EXAMPLE 5

In Vitro Interaction of Homer Proteins with Intracellular

Inositol Trisphosphate Receptors

To demonstrate that Homer proteins interact in vivo with inositol trisphosphate receptors immunoprecipitation studies were performed using brain tissue. Rats or mice were sacrificed by decapitation and the cerebella were dissected

immediately. Cerebella were sonicated in TE buffer (50 mM Tris, 1 mM EDTA, pH 7.4) containing 1% CHAPS and protease inhibitor cocktail (~100 mg wet weight/ml). The homogenate was centrifuged at 90,000 rpm, 20 m, 4°C in a TLA 100.3 rotor. 100 µl of the cerebellar extract was used for each
5 immunoprecipitation assay with the following antibodies: 3 µl of crude Homer 1, Homer 2 or Homer 3 antibodies (Xiao *et al.*, in press); 20 µg of affinity purified inositol trisphosphate antibody (gift from Alan Sharp). Antibodies and extract were incubated for 30 m at 4°C, then 60 µl of 1:1 protein A or protein G (for goat antibody) sepharose slurry was added. The antibody/extract/beads were
10 incubated for an additional 90 m at 4°C. After washing 3x10 m in TE-CHAPS buffer, the proteins were eluted from the beads with 30 µl of 4% SDS loading buffer and analyzed by SDS-PAGE and immunoblot.

Results from these studies showed that the inositol trisphosphate receptor specifically co-precipitates with antisera directed against Homer 1, Homer 2 and
15 Homer 3.

EXAMPLE 6

Calcium Mobilization is Decreased by Transient Expression of Homer Protein Without a Coiled-Coil Domain

20 To demonstrate that Homer cross-links metabotropic glutamate receptors and inositol trisphosphate receptors to provide or enhance a functional signaling complex, calcium mobilization was examined in cells transient expressing truncated forms of Homer protein. The truncated Homer protein used lacks the coiled-coil domain and is unable to form a bridge linking the mGluR at the cell surface with intracellular
25 inositol trisphosphate receptors. The truncated form of Homer protein resembled Homer 1a with the exception of 11 residues at the carboxy-terminal. This form of Homer results in enhanced expression of Homer protein as compared with transfection of Homer 1a in heterologous cells. The Homer protein was introduced into Purkinje cells in primary cerebellar cultures and glutamate induced effects on
30 calcium mobilization was measured.

Embryonic mouse cerebellar cultures were prepared and maintained according

to the method of Schilling *et al.* (Schilling *et al.*, *Neuron* 7:891 1991). At 4-5 DIV, cultures were transfected with plasmids coding for E-GFP (Clontech) and either full-length Homer 1b or an IEG form of Homer 1. The IEG form of Homer 1 was a 186 amino acid amino-terminal fragment of Homer 1b. Plasmids were purified by cesium banding. Three combinations of the plasmids were transfected. Group I (control), 20µg of E-GFP and 40µg of pRK5 vector; group II, 20µg of E-GFP and 40µg of pRK5 Homer 1 IEG; group III: 20µg of E-GFP and 40µg of pRK5 Homer 1b. Plasmid DNA was mixed with gold particles (0.6 micron), and coated onto plastic tubing. DNA was then ballistically transfected into cells according to the manufacturer's protocol (Helios Gene Gun System, BIO-RAD). After transfection, cultures were returned to the incubator and maintained for an additional 2 days for a total of 7-8 DIV at the time of use for imaging experiments.

Patch electrodes were attached to the somata of GFP-expressing Purkinje cells and a holding potential of -60 mV was applied. Micropressure electrodes (1µm tip diameter) were filled with quisqualate (100 µm in external saline) and were positioned ~20 µm away from large-caliber dendrites. Test pulses were delivered using positive pressure (6 psi, 1 sec). Cells were bathed in a solution that contained (in mM) NaCl (140), KCl (5), EGTA (0.2), MgCl₂ (0.8), HEPES (10), glucose (10), tetrodotoxin (0.005), and picrotoxin (0.1), adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. The recording electrode contained CsCl (135), HEPES (10), fura-2 K₅ salt (0.2), and Na₂-ATP (4), adjusted to pH 7.35 with CsOH. Patch electrodes yielded a resistance of 3-5 MΩ when measured with the internal and external salines described above.

Fura-2 ratio imaging of intracellular free Ca²⁺, was accomplished by measuring the background corrected fluorescence ratio at 340 and 380 nm excitation using a cooled CCD camera system, as previously described (Linden *et al.*, *J Neurosci* 15:5098 1995). Exposure times were 200 msec per single wavelength image. Experiments were conducted at room temperature. Enhanced GFP is weakly excited by illumination in the 380-400 nm spectrum. Based upon the bandpass characteristics of our 340HT15 and 380HT10 excitation filters and the absorption spectrum of enhanced GFP (Clontech), we estimate that <1% of the signal at 340 nm excitation

and <5% of the signal at 380 nm excitation is contributed by GFP, even in those cells where the fura/GFP loading ratio is smallest. This could lead to a small (<5%) systemic underestimation of free calcium concentration that should distribute randomly across experimental groups.

- 5 Calcium mobilization in the absence of influx was measure by ratio imaging fura-2 in Purkinje cells bathed in Ca^{+2} -free external saline and stimulated with a micropressure pulse of quisqualate, a metabotropic glutamate receptor agonist (Linden, *Neuron* 17:483 1996). The resultant Ca^{+2} transient is triggered by an mGluR and inositol trisphosphate pathway since it is completely blocked by either an mGluR
- 10 antagonist ((+)-MCPG, 500 μM in the bath) or a novel specific inositol trisphosphate receptor-associated ion channel blocker, xestospongin C (1 μM in the internal saline). Purkinje cells transfected with a truncated form of Homer showed mGluR-evoked Ca^{+2} responses with a decreased amplitude (170 ± 9 nM, mean \pm SEM, n=30 cells) and an increased latency (10.5 ± 1.8 sec) as compared with cells transfected with
- 15 Homer 1b (244 ± 17 nM, 4.2 ± 0.9 sec, n=23) or an empty vector control (239 ± 19 nM, 4.5 ± 1.1 sec, n=15). The decay phase of the Ca^{+2} response appeared somewhat slower in neurons transfected with the truncated form. While the total Ca^{+2} flux appeared similar in cells transfected with truncated and complete Homer proteins and in empty vector controls, the measurement could not be made because the tail of the
- 20 Ca^{+2} response was abbreviated due to the constraints of the image buffer capacity.

EXAMPLE 7

Determination of the Crystal Structure of Homer Protein

The crystal structure of Homer protein and a Homer protein binding site were determined. Results of these experiments are presented in Table

25 (a) Protein Expression and Purification

- Residues 1-120 of rat Homer 1a were expressed in *Escherichia coli* BL21 cells as a C-terminal fusion to glutathione-S-transferase (GST-laEVH) as previously described (Tu *et al.*, *Neuron* 21:717 1998). Selenomethionine-substituted (SeMet) GST-laEVH was prepared by expression in the methionine auxotrophic strain B834
- 30 (DE3) (Novagen). 5 mL of an overnight culture grown at 37°C in LB media

supplemented with 100 µg/mL ampicillin (Sigma) was added to 4L M9 minimal media (Gibco BRL) supplemented with 100 µg/mL ampicillin, 0.05 mg/mL alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, tyrosine, L-selenomethione, 1 µM thiamine (Sigma), 2mM MgSO₄, 1% glucose, 100 µM CaCl₂. Cells were grown to an A₆₀₀ of 0.5 at which time IPTG (Calbiochem) was added to a final concentration of 0.2 mM. Cells were grown for an additional 3 hours, harvested by centrifugation, and resuspended in 1x PBS/1% Triton. Pepstatin A and leupeptin (Boehringer-Mannheim) were added to a final concentration of 1 µg/mL, and PMSF (Life Technologies) was added to 0.5 mM. Cells were lysed by sonication and centrifuged at 13,000 rpm in an SS-34 rotor to pellet cell debris. The cleared lysate was added to a 5 mL glutathione-agarose (Sigma) column. The column was washed in succession with twenty column volumes of 1x PBS/1% Triton, twenty column volumes of 1x PBS, and ten column volumes of cleavage buffer (50 mM Tris [7.4], 150 mM NaCl, 2.5 mM CaCl₂, 50 mM β-mercaptoethanol). All buffers were degassed. A 50% slurry of glutathione-agarose beads loaded with fusion protein was incubated with 20 U of biotinylated Thrombin (Novagen) for 16 h at room temperature. The released cleavage product (Ia-EVH) was collected, and the biotinylated Thrombin was removed with streptavidin-agarose beads (Novagen). Ia-EVH was further purified by cation-exchange chromatography using a Resource S column (Amersham-Pharmacia).

(b) Crystallization and Data Collection

Crystals of native and SeMet protein were grown in hanging drops by the method of vapor diffusion (Wlodawer *et al.*, *Proc Natl Acad Sci USA* **72**:777 1975). 1 µl of a 9 mg/mL native or SeMet protein solution was mixed with a 1:1 dilution of reservoir buffer (30% PEG 3350, 87 mM MgSO₄, 50 mM HEPES, pH 7.3) with distilled water and equilibrated over 1 mL of reservoir buffer. All crystallization trials for the SeMet protein were set up under anaerobic conditions to minimize potential problems due to oxidation. Two different crystal forms were observed for both the native and the SeMet protein. Crystals in the orthorhombic space group P2₁2₁2₁, (unit cell dimensions a = 33.79 Å, b = 51.40 Å, c = 66.30 Å) typically grew to a size of 0.5

mm x 0.03 mm x 0.03 mm. Crystals in the trigonal space group $P3_221$ (unit cell dimensions $a = b = 49.94 \text{ \AA}$, $c = 80.91 \text{ \AA}$) grew to a size of 0.4 mm x 0.1 mm x 0.1 mm. All data used for phasing and refinement were collected from a single trigonal SeMet crystal soaked in mother liquor plus 10% (v/v) ethylene glycol for approximately three minutes prior to flash freezing in a gaseous nitrogen stream at -180°C. X-ray diffraction data suitable for multiwavelength anomalous dispersion (MAD) phasing were collected at four wavelengths at or near the Se absorption edge. These data were collected at beamline X4A of the National Synchrotron Light Source at Brookhaven National Laboratory using an R-Axis IV image plate detector. Nonoverlapping oscillations (2°) at ϕ and $\phi + 180^\circ$ were measured over a 90° rotation of the crystal, interleaving the four wavelengths. All data were processed and scaled using the DENZO/SCALEPACK programs (Otwinowski and Minor, *Meth Enzymol* 276:307 1997). Data collection statistics are shown in Table 1.

(c) Structure Solution and Refinement

The expected two selenium sites were determined and refined using the program SOLVE (Terwilliger and Berendzen, *Acta Crystallogr* D53:571 1997; Terwilliger and Eisenberg, *Acta Crystallogr* A39:813 1983) and initial Se scattering factors from (Hall *et al.*, *Cell* 91:85 1997). Values for the refined Se scattering factors as determined by SOLVE are shown in Table 1. The electron density maps calculated with the experimental MAD phases as determined by SOLVE were improved by solvent flattening and histogram matching using DM (Collaborative Computational Project, 1994). An initial model of residues 1-105 was built into 1.8 Å experimental electron density maps using the program O (Jones *et al.*, *Acta Crystallogr* A47:110 1991). After one round of simulated annealing with bulk solvent correction and positional and B-factor refinement using CNS (Brünger *et al.*, *Acta Crystallogr* D54:905 1998), residues 106-111 were built into $2F_o - F_c$ maps. The model was refined against the maximum-likelihood target (Pannu and Read, *Acta Crystallogr* A52:659 1996) using data to 1.7 Å Bragg spacing collected at 0.9879 Å. Eight rounds of model building and water addition alternated with B-factor and positional refinement yielded the current model, which includes residues 1-111 and 88 water molecules. No electron density was observed for residues 112-120. This model has a

crystallographic R value of 25.3% and a free R value of 28.4%. The solvent content is ca. 40.6%, with one molecule per asymmetric unit. Fractional solvent accessibility for each residue was calculated in X-PLOR (Brünger, X-PLOR, Version 3.1: A system for X-ray crystallography and NMR (New Haven, CT: Yale Univ. 1992).

5 **(d) Determination of Homer Site by Site Directed Mutagenesis**

Point mutants of N-terminally myc-tagged, full-length Homer 1b and 1c and Homer 1 EVH1 were made using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Expression constructs were transiently transfected into HEK293 cells using calcium phosphate methods. About 24-48 h post-transfection, cell lysates were
10 prepared in 1x PBS/1% Triton X-100 (Sigma) and protease inhibitors. GST pull-down assays were performed by mixing 100 µl of cell lysate with GST-mGluR5 or GST-Shank3 (residues 1143-1408) (Tu *et al.*, in press) bound to glutathione-agarose, incubating at 4°C for 2 h, and washing with 1x PBS and 1x PBS/1% Triton X-100. Bound products were eluted with 100 µl 2x SDS loading
15 buffer and detected by SDS-PAGE and immunoblot using anti-myc antibody 9E10 (Invitrogen) and ECL reagents (Amersham).

EXAMPLE 8

**Homer Expression is Upregulated in Certain Brain Regions in Response to
Electrically Induced Seizures**

20 Rat Homer 1a was cloned based on its rapid upregulation in hippocampal granule cell neurons following electrically-induced seizure (MECS; see Brakeman *et al.*, *Nature* **386**:284 1997) The expression of other members of the Homer family was examined in the brain following seizure. Radio-labeled riboprobes were prepared using unique sequences for Homer 1a, Homer 1b, Homer 1c, Homer 2a, Homer 2b
25 and Homer 3. Probes used did not distinguish between the splice forms of Homer 1b and 1c or Homer 2 and 2b.

(a) In Situ Hybridization

Anti-sense and sense cRNA probes were generated from each mouse Homer

plasmid by in vitro transcription in the presence of [³⁵S]UTP, as previously described (Lyford *et al.*, *Neuron* 14:433 1995). Probe for Homer-1a (Xiao, 1998; GenBank #) was derived from nucleotides 1342 to 2140, for Homer 1b/c (Xiao, 1998; GenBank #) from nucleotides 785 to 1396, for Homer-2a/b (Xiao, 1998; GenBank #) from nucleotides 486 to 1561, and for Homer-3 (GenBank #) from nucleotides 371 to 2123. Probe (about 10⁶ cpm in 75 µl hybridization buffer) was applied to each slide. Coverslipped slides were then incubated in humidified chambers overnight at 56°C. Following completion of wash steps, slides were air dried and exposed to Kodak Biomax MR film for 2-3 days.

The anatomic distribution in unstimulated animals reveals that expression of Homer 1a is similar to the expression of Homer 1b and Homer 1c. High levels of expression of Homer 1a are observed in the hippocampus, striatum and cortex. In the cortex, there is laminar expression with the highest levels in the superficial and deep layers. Expression of Homer 2a and 2b is enriched in the thalamus, olfactory bulb and principle neurons of the hippocampus in contrast to the cortex where low levels of expression of Homer 2a and 2b are observed. Homer 3 is expressed primarily in the cerebellum and hippocampus.

In situ hybridization studies demonstrate the dramatic induction of Homer 1a in response to MECS. In the hippocampus, induction of expression is estimated to be greater than 20-fold compared to hippocampus from unstimulated animals. MECS induced an increase in Homer 1b and 1c expression of about 1.5 fold as determined by blot analysis. Expression of Homer 2 and Homer 3 is not altered in response to MECS.

25

EXAMPLE 9

Formation of Multimeric Complexes of Homer Proteins.

The CC secondary structure is implicated in protein-protein interactions (Lupas, 1996 *supra*). Therefore, the possibility that this domain might confer the ability to form homo- or hetero-multimers between Homer family members was

examined. For examining the coiled-coil interaction of Homer family members, myc-tagged Homer-1c and Homer-2b were transfected into HEK293 cells and cell extracts were made 2-3 days post-transfection. Cell lysates were treated as described above.

5 First, the ability of full length, bacterially-expressed GST fusion proteins of Homer to bind full length myc-tagged Homer proteins expressed in HEK293 cells was tested. myc Homer 1c bound Homer 1b, Homer 2b, Homer 3, Homer 1b and Homer 2b carboxy-terminal CC domain, but not Homer 1a or Homer 2-amino-terminus. This is consistent with the notion that the CC domain is important in the interaction, since
10 Homer 1a and Homer 2-amino-terminus do not encode the CC domain. To test the specificity of the CC domain interactions, GST fusions of dynein IC-1a and dynein IC-2c were generated. The CC domains of these proteins show modest sequence to Homer family CC domains and bind to the CC domain of dynactin (Gill, 1991 *supra*). None of the myc-tagged Homer family members bound to either dynein IC-1a or
15 dynein IC-2c.

To determine whether Homer family members naturally form multimers in brain, immunoprecipitates of cerebellum were examined. Extracts immunoprecipitated with Homer 1b/c antibody contained Homer 3, while extracts immunoprecipitated with Homer 3 contained Homer 1b/c. While it is possible that
20 these co-immunoprecipitated Homer family members are associated by means other than their CC domains, the fact the amino-terminus of Homer is monovalent and cannot form extended concatomers supports a model of multimerization mediated by the CC domains. Homer 2 was not detected as a multimer with either Homer 1 or Homer 3 in these immunoprecipitation experiments.

25

EXAMPLE 10

Homer Family Proteins are Enriched at Brain Synaptic Fractions and are Expressed in Certain Peripheral Tissues

The distribution and localization of Homer family proteins was examined at
30 the using immunochemical methods. Tissue extracts were assayed using immunoblot

analysis and tissue localization was examined using immunohistochemistry at the light and ultrastructural levels.

(a) **Immunoblot Analysis** Immunoblot staining of SDS (2%) extracts of various brain regions were examined to assess the distribution of Homer proteins in the brain. Homer 1b/c antibody detected a single band of about 47 kDa in cortex, hippocampus and cerebellum. These regions have similar levels of expression. The Homer 2a/b antibody detected a single major band in each of cortex, hippocampus and cerebellum. Less intense, higher apparent molecular mass bands were detected at about 60 and about 80 kDa. Homer 3 immunoblots showed low level expression in cortex and hippocampus and intense staining of a single band in cerebellum (47 kDa). Immunostaining was completely blocked by preincubating the antibody with 10 µg/ml of the relevant peptide antigen.

(b) **Immunohistochemistry**

For light microscopy, rats were deeply anesthetized with sevoflurane and perfused through the aorta with 250 ml of saline followed by 400 cc each of 4% paraformaldehyde in 0.1% phosphate buffer (pH 6.5) and 4% paraformaldehyde in 0.1% phosphate buffer (pH 8.5). The rat was allowed to postfix for 1 hr. at room temperature and then perfused with 15% sucrose in 0.1% phosphate buffer (pH 7.4). The brain was removed and sectioned at 40 µm on a freezing sliding block microtome and collected in PBS. Tissue was stained with an immunoperoxidase technique, as follows. Brain sections were incubated in PBS containing 0.3% H₂O₂ and 0.25% Triton X-100 for 30 m and then washed 3x5 m in PBS. Sections were incubated in a buffer "PGT" containing 3% normal goat serum (Colorado Serum Co.) and 0.25% Triton X-100 in PBS for 1 hr. and then transferred to the primary antiserum diluted 1:750 in the same PGT buffer. Sections were gently shaken for 48 h at 4°C, washed 4x5 m in PBS and then incubated for 1 hr. at room temperature in a goat anti-rabbit IgG conjugated to horseradish peroxidase (Biosource International) diluted 1:100 in PGT. Sections were washed 4x5 m in PBS and incubated for 6 m at room temperature in 0.05% diaminobenzidine dihydrochloride (DAB:Sigma) and 0.01% H₂O₂ in 0.1 M phosphate buffer. Sections were washed in PBS, mounted onto

gelatin chrome-alum subbed slides, dehydrated in a series of graded ethanol, cleared in xylene and coverslipped with DPX (BDH Limited).

Immunohistochemistry was performed to determine the cellular localization of Homer 1b/c and Homer 2a/b and Homer 3 in rat brain. Light microscopic examinations indicated that all three Homer proteins are enriched in Purkinje neurons. Immunoreactivity is present in the cytoplasmic region of the soma and extends prominently into the dendritic arbor. The nucleus is not stained. Little or no staining is detected in the contiguous granule cell layer. A similar light microscopic pattern of cellular localization was detected for Homer 3. Homer 2 immunostaining in cerebellum also showed staining in Purkinje neurons, but appeared technically less differentiated.

(c) Electron Microscopy

For EM, a postembedding immunogold method as described previously (Wang, *et al.*, *J Neurosci* 18:1148 1998) was used and modified from the method of (Matsubara, *et al.*, *J Neurosci* 16:4457 1996). Briefly, male Sprague-Dawley rats were perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M phosphate buffer. Two hundred micrometer parasagittal sections of the rostral cerebellum (folia III-V) were cryoprotected in 30% glycerol and frozen in liquid propane in a Leica EM CPC. Frozen sections were immersed in 1.5% uranyl acetate in methanol at -90°C in a Leica AFS freeze-substitution instrument, infiltrated with Lowicryl HM 20 resin at -45°C, and polymerized with UV light. Thin sections were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline/0.1% Triton X-100 (TBST), followed by 10% normal goat serum (NGS) in TBST, primary antibody in 1% NGS/TBST, 10 nm immunogold (Amersham) in 1% NGS/TBST plus 0.5% polyethylene glycol, and finally staining in uranyl acetate and lead citrate. Primary antibodies were used at dilutions of 1:500 for Homer 1b and 1:100-1:400 for Homer 3.

Immunogold EM of Purkinje neurons of the cerebellum was performed to determine whether Homer family proteins are associated with synaptic structures. Homer 1b/c showed striking localization to the region of the postsynaptic spine. Gold particles are densely concentrated in the region of the postsynaptic density (PSD). A

very similar distribution is noted for Homer 3 immunoreactivity. It is noted that rather than being concentrated directed over the PSD or the contiguous plasma membrane, the majority of the gold particles appear to be present in the cytoplasm immediately subjacent to these structures.

5 **Peripheral Tissues** Homer proteins are expressed in peripheral tissues. In detergent extracts of heart and kidney, a single band at 47 kDa immunoreactive to Homer 1b and 1c is detected. In extracts of liver, a complex of three bands ranging from about 44 to 47 kDa is detected. In heart, liver, skeletal muscle and intestine, bands immunoreactive to Homer 2a and 2b are detected. Homer 3 immunoreactive
10 bands are detected in extracts of lung and thymus.

Subcellular Distribution To examine the subcellular distribution of Homer proteins, a biochemical fractionation of rat forebrain was performed and fractions were analyzed by Western blotting with Homer antibodies. Fractions were blotted for mGluR5, BIP and synaptophysin to monitor anticipated enrichment of
15 fractions. Homer 1b/c, 2a/b and 3 were present in the crude nuclear pellet (P1), the medium spin crude synaptosomal pellet (P2), and the high speed microsomal pellet (P3). BIP is a 78 kDa ER resident protein (Munro and Pelham, *Cell* 48:899 (1987)), and was enriched in both the P3 and the S3 fractions. While Homer 1b/c and Homer 3 were not abundant in the soluble (S3) fraction, Homer 2 was enriched in the S3
20 fraction. The P2 fraction was subfractionated after hypotonic lysis. The 25,000 x g pellet (LP1), which is enriched in PSDs (Huttner *et al.*, *J Cell Biol* 96:1374 (1983)), showed enriched presence of mGluR5. The high speed pellet (165,000 x g; LP2) showed the anticipated enrichment in the synaptic vesicle protein synaptophysin (P38). Each of the Homer proteins was enriched in the LP1 fraction relative to LP2.
25 the final soluble fraction (LS2) was uniquely enriched in Homer 2.

EXAMPLE 11**Transgenic Mouse Model Demonstrates that Expression of Homer 1a Selectively Blocks Binding of Homer 1b/c to mGluR5 In Vivo**

N-terminal myc-tagged full-length Homer 1a ORF was cloned into the expression vector pT2 (Gordon, *et al.*, *Cell* 50:445 1987; Aigner, *et al.*, *Cell* 83:269 1995). Transgenic mice were generated at the University of Alabama Transgenic Facility. Expression of the transgene protein was assayed by western blot with rabbit polyclonal antisera that recognizes all Homer 1 isoforms (pan-Homer 1 antibody) and myc antibody.

Homer 1a is unique within the family of Homer related proteins in that it is dynamically regulated and it lacks the CC domain. Accordingly, it was hypothesized that the IEG would bind to group 1 metabotropic receptors and disrupt the formation of multivalent complexes of Homer and mGluR. To examine this hypothesis, a transgenic mouse was generated that expresses Homer 1a under the control of a modified Thy-1 promoter (Gordon *et al.*, 1987, *supra*), which drives neuron-specific expression in postnatal brain (Aigner *et al.*, 1995, *supra*). Transgenic mice expressed Homer 1a at high levels in cortex, hippocampus, cerebellum and thalamus/brainstem relative to levels in wild type litter mate controls. The pattern of Homer 1a transgene expression is consistent with the previously reported activity of this promoter (Gordon *et al.*, 1987, *supra*). As expected, antibodies for both Homer 1b/c and Homer 2a/b co-immunoprecipitated mGluR5 from detergent extracts of wild type forebrain. By contrast, Homer 1b/c antibody did not co-immunoprecipitate mGluR5 from transgenic mice. The effect of Homer 1a transgene expression was selective in that it did not disrupt the co-immunoprecipitation of Homer 3 with Homer 1b/c. The latter observation is consistent with the notion that the Homer 1b/c-Homer 3 interaction is mediated by the CC domain and is predicted not to be altered by Homer 1a expression. Homer 1a was not part of the complex co-immunoprecipitated with Homer 1b/c, consistent with the notion that the CC is necessary for association with the complex. The effect of the Homer 1a transgene in blocking the in vivo coupling of mGluR5 and Homer 1b/c was additionally selective in that Homer 2 antibody co-

immunoprecipitated mGluR5 similarly from extracts of wild type and transgenic mice. Thus Homer 1a appears to selectively disrupt the interaction of Homer 1b/c with mGluR5 but not Homer 2 with mGluR5. Homer 3 is less highly expressed in forebrain than Homer 1b/c or Homer 2a/b and co-immunoprecipitates of mGluR5 with Homer 3 antibody were less clean. Accordingly, it could not be determined in these experiments whether Homer 1a also competes with Homer 3. Identical results were obtained in tow independent mouse lines that express Homer 1a transgene. The Homer 1a expressing transgenic mice have not been behaviorally characterized but appear normal in size and gross motor activity.

EXAMPLE 12

Yeast Two-Hybrid Screen

To examine the physiological functions of Homer, a novel family of proteins was identified based on its ability to interact with Homer family proteins in a yeast two-hybrid screen of a brain cDNA library. Homer 1a was subcloned into pPC97 (Chevray and Nathans, *Proc. Natl. Acad. Sci. U.S.A.*, 89:5789 (1992)) and used to screen a random primed cDNA library prepared from seizure-stimulated rat hippocampus and cortex cloned in pPC86 (Chevray and Nathans, 1992, *id.*) as described previously (Brakeman *et al.*, *Nature*, 386:284 (1997)). The same library was rescreened using the PDZ domain of Shank 3 (amino acid residues 559-673) cloned into pPC86. The Shank 3 PDZ domain was also tested for interaction with mGluR constructs in pPC86. mGluR5 constructs included a wild type C-terminal 241 amino acid fragment and a four amino acid carboxy-terminal deletion of the same fragment.

Using Homer as "bait" in a yeast two-hybrid screen of a rat cortex and hippocampus cDNA library, multiple cDNA isolates of two novel genes were obtained. Sequencing and full length cloning identified these as distinct members of a gene family, termed Shank 1 and 3 (Naisbitt *et al.*, in press). Shank family proteins are closely related to a previously described protein, termed Cortactin Binding protein (CortBP-1; Du *et al.*, *Mol. Cell. Biol.*, 18:5838 (1998)).

EXAMPLE 13

Interactions Between Homer Proteins and Shank Proteins

In Vitro and In Vivo

To characterize the interaction between Homer proteins and Shank proteins,
5 the Shank cDNAs isolated from the yeast two-hybrid screen (Example 10)) were
expressed in HEK293 for GST pulldown assays with GST-Homer 1a. The interaction
between Homer and Shank proteins was further characterized by co-
immunoprecipitation assays.

(a) Expression Constructs

10 Shank expression constructs were prepared as described (Naisbitt *et al.*, in
press). Site directed point mutants of Shank were generated using Quik Change
(Stratagene). GST fusion constructs were prepared by polymerase chain reaction
(PCR) using Pfu Polymerase (Stratagene) with specific primers that included Sall and
NotI sequences. After digestion with Sall/NotI, PCR products were subcloned into
15 pGEX4T-2 vector (Pharmacia Biotech, Uppsala, Sweden) or N-myc-tagged pRK 5
vector (modified from Genentech). All constructs were confirmed by sequencing.
GST-fusion proteins were expressed in BL21 E. coli strains (GIBCO, BRL). Bacteria
were harvested and lysed in PBS, 1% Triton X-100, 2 mM phenylmethylsulfonyl
fluoride (PMSF) and pelleted at 13,000 rpm (Sorvall SS-34) at 4°C for 5 m. Proteins
20 were purified by incubating 1 ml bed volume glutathione-sepharose beads (Sigma)
with bacterial supernatant at 4 °C for 10 m. and washed twice with PBS and PBS plus
1% Triton X-100. Bound proteins were eluted with 10 mM glutathione and dialyzed
against PBS at 4°C. Protein concentrations were measured by BCA (Pierce, Illinois).
mGluR5 constructs and mutants are described in Tu *et al.*, *Neuron* 21:717 (1998).

25 (b) GST pulldown and Co-immunoprecipitation Assays

Expression constructs were transiently transfected into HEK293 cells using
the calcium phosphate method. Cells were lysed 24-48 h post-transfection with PBS
plus 1 % Triton X-100. GST pull down assays were performed by mixing 100 µl cell
lysates with beads charged with GST fusion proteins (1-3 µg/50 µl bed vol.) at 4°C for

2 h followed by washing once with PBS, once with PBS plus 1% Triton X-100. Bound proteins were eluted with 100 μ l 2 x SDS loading buffer and detected by SDS-PAGE and immunoblotting using ECL reagents (Amersham). GST pull down assays of mGluR1a and mGluR5 from brain lysates were performed by sonicating rat
5 cerebellum or cortex in 50mM Tris, 1mM EDTA, 1% CHAPS (Sigma), 0.5% deoxycholic acid (Sigma) and proteinase inhibitors with GST-proteins and these tissue extracts were then processed as above. For immunoprecipitation from COS7 cells, transfected cells were extracted in RIPA (see Naisbitt *et al.*, in press). Soluble extracts were precipitated with 2 μ g control non-immune IgG, Myc or Shank 1 (56/e)
10 antibodies (Naisbitt *et al.*, in press).

Extracts of forebrain crude synaptosomes for immunoprecipitation were prepared using deoxycholic acid as described previously (Dunah *et al.*, *Mol. Pharmacol.* 53429 (1998)). Forebrain P2 fraction was extracted in 1% deoxycholic acid, dialyzed over night into 0.1% Triton X-100, 50 mM Tris, pH 7.4. Concurrently,
15 5 g of each antibody was pre-incubated overnight with 10 μ l bed volume protein A-sepharose. After centrifugation at 100,000g for 1 h, 50 μ g of extract was incubated with antibody-protein A in 100 μ l 0.1% Triton X-100, 50 mM Tris, pH 7.4 for 2 h at 4°C. Pellets were washed 4 times with 1 ml incubation buffer, and bound proteins were analyzed by immunoblotting.

20 **Antibodies** Shank antibodies were raised in rabbits immunized with GST-fusions of Shank 3 residues 1379-1740 and 1379-1675 (Covance, Denver, PA). Similar bands were seen on rat brain immunoblots with both antisera. GKAP, PSD 95 and Shank 1 (56/e) antibodies are described in (Naisbitt *et al.*, in press). Homer antibodies are described above. Anti-mGluR 1a monoclonal antibody is from
25 Pharmingen and rabbit polyclonal mGluR5 antiserum was obtained from Dr Richard Huganir (Johns Hopkins University).

Shank cDNAs derived from the yeast two-hybrid screen were expressed in HEK293 cells for GST pulldown assays with GST-Homer 1a. Each of the Shank polypeptides specifically bound Homer 1a. Based on the finding that the Homer
30 EVH1 domain binds a specific proline-rich motif, three potential Homer binding sites (or Homer "ligands") that are conserved in Shank 1, 2, 3 and CortBP-1 were

identified.(Naisbitt *et al.*, in press). To define the Homer binding site on Shank family proteins, three deletion fragments of Shank 3 that included, respectively, amino acid residues 559-908, amino acid residues 1143-1408, and amino acid residues 1379-1740 were testing for their ability to bind to Homer 1b, Homer 1c,
5 Homer 2 and Homer 3 in GST pulldown assays. Similar binding specificity was detected with each of the Homer proteins. Only Shank3 fragment 1143-1408 bound to Homer. This region contains the amino acid sequence that most closely resembles the Homer ligand peptide consensus (LVPPPEEFAN; residues 1307-1316). A similar sequence is present in Shank1 (PLPPPLEFSN 1563-1572; see Naisbitt *et al.*,
10 in press). CortBP possesses two similar sites; (PLPPPLEFAN; residues 813-822) and (FLPPPESEFDA residues 878-887). Fragments of Shank3 containing amino acid residues located nearer the amino-terminal of the protein such as Shank 3 fragment 559-908 (which includes the PDZ domain and the first proline-rich motif) did not bind to Homer, but did bind to GKAP (Naisbitt *et al.*, in press). Similarly, Shank3
15 fragment 1379-1740, which includes the carboxy-terminal proline-rich sequence and the SAM domain, did not bind to Homer, though it is capable of binding itself and cortactin (Naisbitt *et al.*, in press). These studies identify the Homer binding site as being distinct from either the PDZ domain that binds GKAP, or the proline-rich binding site that binds cortactin and which is located nearer to the carboxy-terminal
20 (Naisbitt *et al.*, in press).

To confirm the site of Homer interaction, site directed point mutants of the putative Homer ligand in Shank3 were assessed for their ability to bind to GST-Homer 1c. Full length wild type Shank 3, Shank3(P1311L), and Shank3(F1314C) were expressed in HEK293 cells and assayed for binding to GST-
25 Homer 1c. Compared to wild type Shank 3, both point mutants showed dramatically reduced binding to Homer., These experiments provide further confirmation that the Homer ligand in Shank3 is the principle site of interaction.

It has been previously demonstrated that amino acids 1-110 of the Homer EVH1 domain are necessary and sufficient for binding to Homer ligands (Brakeman
30 *et al.*, 1997, *supra*; Tu *et al.*, 1998, *supra*). To confirm that the EVH1 domain of Homer mediates interactions with Shank, a series of point mutants of the Homer 1

EVH1 domain were generated. Mutations that disrupted binding to mGluR5 disrupted binding to Shank 3 in an identical manner, indicating Homer binds both proteins via a similar EVH1-dependent mechanism (Beneken *et al.*, in press).

To confirm the interaction between Homer and Shank in a mammalian cell context, co-immunoprecipitation experiments were performed in heterologous cells. COS7 cell were transfected with Myc tagged-Homer 1b, Shank 1, or Shank 1 plus myc-Homer 1b. Detergent extracts of cells were subjected to immunoprecipitation and blotted with myc, shank, or control (non-immune IgG) antibodies. Homer 1b was used in these experiments because it expresses more efficiently in mammalian cells than Homer 1a. There is co-immunoprecipitation of Homer with Shank antibody and of Shank with myc antibody only from cells expressing *both* Shank and myc-Homer 1b.

To demonstrate the *in vivo* relevance of the Homer-Shank interaction, co-immunoprecipitation experiments were performed using detergent extracts of rat brain. Detergent extracts of rat forebrain fractions were immunoprecipitated with Shank and control (non-immune) antisera. Immunoprecipitates were blotted for Homer, Shank and GRIP antibodies. Antibodies raised against a fusion protein of Shank 1 immunoprecipitated Homer 1b and 1c proteins as well as Shank from rat forebrain. GRIP was not co-immunoprecipitated with Shank and neither Shank or Homer were precipitated by non-immune IgGs. Furthermore, another Shank antibody, generated against Shank 3 fragment 1379-1675, co-immunoprecipitated Homer 1b and 1c extracted from both cerebellum and cortex.

EXAMPLE 14

Homer and Shank Mediate Clustering of Cell-Surface Receptors

Shank proteins may link Homer proteins with components of a cell-surface clustering complex, such as the NMDA clustering complex.

COS7 cells were transfected using the Lipofectamine method (GIBCO-BRL) on poly-lysine coated coverslips for clustering experiments, as described in Naisbitt *et*

al. (in press) and Kim *et al.* (*Neuron* 17:103 1996). Primary antibodies were used as follows: GKAP C9589, 1 µg/ml (Naisbitt *et al.*, in press); Shank 56/e 0.5 µg/ml (Naisbitt *et al.*, in press), PSD-95, 1:1000 diluted guinea pig serum (Kim *et al.*, *Neuron* 378:85 1995). Cy3 and (fluorescein isothiocyanate conjugate (FITC)-
5 conjugated secondary antibodies (Jackson ImmunoResearch) were used at dilutions of 1:500 and 1:100 respectively.

Yeast two-hybrid screens were performed as described in Example 10.

A yeast two-hybrid screen of the same rat brain cDNA library was performed using the PDZ domain of Shank3 as bait. From this screen, two identical clones of
10 the carboxy-terminus of GKAP-3/SAPAP3 were isolated. In a reciprocal screen, Naisbitt *et al.* (in press) isolated multiple clones of Shank1, 2 and 3 using GKAP as bait. This result provides independent confirmation of the specificity of the interaction between the Shank and GKAP/SAPAP families of proteins.

The cDNA from the yeast two-hybrid screen encoding the carboxy-terminal
15 347 amino acids of GKAP-3 was expressed with an amino-terminal *myc* tag in HEK293 cells and tested for binding to GST fusion constructs of Shank3 and other PDZ containing proteins. The GST fusion of Shank3 fragments containing just the PDZ domain (residues 559-673) was sufficient to bind GKAP3, while a Shank3 construct lacking the PDZ domain (residues 665-908) failed to bind. Additionally,
20 PDZ domains of GRIP and SAP102 failed to pull down GKAP3, demonstrating the specificity of the Shank-GKAP interaction.

The above findings suggest that Homer, Shank and GKAP may assemble into a ternary complex. To explore this further, GST pull-down assays were performed using rat brain extracts. The carboxy-terminal 76 amino acids of GKAP1a, containing
25 the Shank PDZ-binding sequence -QTRL, was fused to GST [GST-GKAP(carboxy-terminal)]. GST-GKAP(carboxy-terminal) specifically pulled down both Shank and Homer 1b and 1c, but not GKAP1 or several other proteins (Naisbitt *et al.*, in press). Since GKAP binds directly to Shank but not to Homer (Naisbitt *et al.*, in press), the results suggest that the GKAP pulldown of Homer is mediated by Shank. These
30 findings corroborate the co-immunoprecipitation experiments of Shank and Homer

from brain extracts and confirm that Homer is associated with Shank in a native complex.

Since Shank proteins may link Homer proteins with components of the NMDA clustering complex, co-clustering of these proteins in transfected COS cells was assessed. In cells co-expressing Homer 1b and PSD-95, both proteins showed a diffuse distribution in the cytoplasm. This is not surprising, since Homer and PSD-95 do not interact directly. When cells were transfected with Shank1 and GKAP in addition to Homer and PSD-95, Homer and PSD-95 redistributed into plaque-like clusters in which both proteins were exactly co-localized. By contrast, co-clustering of Homer and PSD-95 was not observed following co-transfection of Homer and PSD-95 with either Shank1 or GKAP alone. Thus, Homer and PSD-95 co-cluster only upon co-expression of Shank and GKAP. Therefore, Shank and GKAP may mediate the formation of a quaternary protein complex containing PSD-95 and Homer (see also Naisbitt *et al.*, in press). Other types of macromolecular complexes may also form when Homer and Shank proteins interact. Cells expressing Homer 1b and Shank 1 (without GKAP or PSD-95) exhibited a redistribution of Homer 1b into a reticular filamentous pattern, as well as into clusters; in both kinds of structures Shank and Homer immunoreactivities were co-localized. These findings provide further evidence for an interaction between Homer and Shank, and suggest that Homer 1b and Shank can co-assemble into higher order macrocomplexes. This result is consistent with the biochemical properties of Shank that include its ability to self-multimerize and bind cortactin (Naisbitt *et al.*, in press). Since Shank, GKAP, and PSD-95 are components of NMDA receptor-associated complex (Naisbitt *et al.*, in press), the identification of Homer as a Shank-binding protein invokes a molecular link between the NMDA receptor complex and Homer-associated synaptic proteins such as mGluR1a and 5 and the inositol trisphosphate receptor.

Group 1 Metabotropic Receptors Based on the observations in heterologous cells that Shank clusters with Homer 1b and that Shank together with GKAP can mediate the co-clustering of Homer and PSD-95, Shank may mediate clustering of group 1 metabotropic glutamate receptors (mGluRs). Co-expression of Shank1 and mGluR5 in COS cells did not result in obvious clustering of either

protein. Similarly, Homer and mGluR5 do not form co-clusters. Co-expression of the three proteins Homer, Shank 1, and mGluR5, however, resulted in conspicuous co-clustering of mGluR5 with Shank 1. Clustering of mGluR5 in these triply transfected cells was dependent on the ability of Homer to bind the receptor since a point mutant
5 of mGluR5 that does not interact with Homer failed to co-cluster with Shank. Thus, both Homer and Shank are required to mediate the clustering of mGluR5.

EXAMPLE 15

The Shank 3 PDZ Domain Binds the Carboxy-Terminus of Group 1 10 Metabotropic Receptors Directly at a Site Distinct from the Homer Binding Site

The Shank PDZ domain shows selective binding to the GKAP carboxy-terminus (Naisbitt *et al.*, *in press*). The carboxy-terminal sequence of GKAP (-QTRL) finds similarities with that of the group 1 mGluRs (mGluR1a -SSSL; mGluR5 -SSTL) and therefore it was determined whether the PDZ domain of Shank
15 can directly bind the carboxy-terminus of group 1 mGluRs. GST-pulldown assays were performed using extracts from heterologous cells expressing a recombinant mGluR5 carboxy-terminal 241 amino acid peptide. The mGluR5 carboxy-terminal tail bound two partially overlapping constructs of Shank 3 that included the PDZ domain (559-908; and 559-673), but not a construct from which the PDZ domain was
20 deleted (amino acids 665-908). Binding of mGluR to the Shank3 PDZ domain was qualitatively similar to mGluR5 binding to Homer 1c and Homer 2. Negative controls included absence of binding of mGluR to SAP102 PDZ1-3 and GRIP PDZ 4-6. Furthermore, a deletion mutant of the mGluR5 polypeptide that lacked the carboxy-terminal four amino acids failed to bind to the PDZ domain of Shank3. Identical
25 interactions between Shank PDZ and mGluR5 C-terminal tail were detected in a yeast two-hybrid analysis. These studies indicate that the PDZ domain of Shank 3 can bind the carboxy-terminus of group 1 metabotropic receptors via a PDZ-mediated interaction with the carboxy-terminal sequence --S S/T L.

To confirm that Shank3 PDZ domain can bind full length native mGluRs,
30 GST pull down assays were performed with detergent extracts of forebrain or

cerebellum. The PDZ domain of Shank 3 bound specifically to mGluR1a and mGluR5 from cerebellum and forebrain, respectively. (Cerebellum predominantly expresses mGluR1, while forebrain expresses predominantly mGluR5.) While it is possible that the Shank3 PDZ pulldown of mGluRs from brain extracts is indirect, via
 5 Shank PDZ pulling down a GKAP-Shank-Homer-mGluR complex, this extended complex is unlikely given the more modest ability of GST-GKAP to pull down Homer.

These studies suggest that Shank may interact with the cytoplasmic tail of mGluR1a/5 both directly, via its PDZ domain, and indirectly, via Homer. The
 10 inability of Shank 1 to cluster mGluR5 in the absence of Homer indicates that the direct PDZ-dependent Shank-mGluR interaction is contingent upon a co-incident Homer interaction. Both modes of interaction with mGluR may be involved in mGluR clustering by Shank and contribute to physiological regulation.

EXAMPLE 16

Shank and Homer Co-Localization at Specific Post Synaptic Densities

Immuno Electron Microscopy A postembedding immunogold method (Petralia *et al.*, *Nature Neurosci* 2:31 1999; Zhao *et al.*, *J Neurosci* 18:5517 1998) was used. Male Sprague-Dawley rats was perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M phosphate buffer (PBS). Parasagittal sections (250 µm) of
 20 the hippocampus were cryoprotected in 30% glycerol and frozen in liquid propane in a Leica EM CPC. Frozen sections were immersed in 1.5% uranyl acetate in methanol at -90 °C in a Leica AFS freeze-substitution instrument, infiltrated with Lowicryl HM 20 resin at -45 °C, and polymerized with UV light. Thin sections were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline/0.1% Triton X-
 25 100 (TBST), followed by incubations in 10% normal goat serum (NGS) in TBST, primary antibody in 1% NGS/TBST, 10 nm immunogold (Amersham) in 1% NGS/TBST plus 0.5% polyethylene glycol, and finally staining with uranyl acetate and lead citrate. For double labeling, the first primary antibody (*e.g.*, Shank; Shank3 1379-1675 antigen) and corresponding immunogold-conjugated antibody (10 nm
 30 gold) were applied, sections were exposed to paraformaldehyde vapors at 80°C for one hour, and the second primary (Homer 1b and 1c) and secondary (20 nm gold; Ted

Pella/BBI International) antibodies were applied the following day. Controls (showing little or no gold labeling) included absence of the primary antibody for single labeling and absence of the second primary antibody for double labeling. Primary antibodies were used at dilutions of 1:100-1:300 for Shank and 1:400 for
5 Homer 1b and 1c.

An antibody generated against a carboxy-terminal region of Shank 3 (amino acids 1379-1675) was used to examine the ultrastructural distribution of the Shank proteins in brain. This antibody recognizes multiple bands on brain immunoblots, including major bands of ~160-180 kD and ~210 kD in forebrain and cerebellum,
10 similar to those seen with other Shank antibodies (see Naisbitt *et al.*, *in press*). The different size bands presumably derive from the multiple Shank genes and splice variants. All Shank immunoreactivity is blocked by incubation of the Shank antibody with the Shank fusion protein antigen.

Immunogold electron microscopy revealed intense Shank immunoreactivity at
15 the PSD of CA1 pyramidal neurons. Gold particles were distributed over the entire region of the PSD. In the same preparations, Homer 1b /1c was found to co-distribute with Shank. In all profiles with immunostaining for both Shank and Homer, gold particles were present over the PSD but also extended into the region subjacent to the PSD. This distribution is similar to the distribution of NMDA receptors associated
20 with the postsynaptic membrane (Petralia *et al.*, 1999, *supra*) and distinct from the distribution of mGluR5 which are most prevalent in the perisynaptic membrane region just outside the PSD (Lujan *et al.*, *Eur J Neurosci* 8:1488 1996). This spatial localization is consistent with the idea that Shank 3 and Homer interact with components of both the NMDA receptor and metabotropic receptor signaling
25 complexes.

This family of proteins that interact with Homer are identical to the Shank family of postsynaptic density (PSD) proteins that interact with GKAP and PSD-95 complex (Naisbitt *et al.*, *in press*). Shank uses distinct domains to bind to GKAP and
30 to Homer, and thus can form a bridge between proteins of this family. Shank/GKAP

is also associated with NMDA receptors through the PSD-complex (Naisbitt *et al.*, in press) and thus the Homer-Shank interaction indicates a molecular link between NMDA receptors and Homer-associated proteins such as mGlu receptors and inositol trisphosphate receptors. This linkage has important implications for the coupling of
5 NMDA receptors to intracellular calcium release pools and for excitatory synapse assembly in general.

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It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

Table 1. Data Collection, Phase Calculation, and Refinement Statistics

MAD Data Collection Statistics				
Wavelength (λ)	09879	09793	09790	09611
Unique reflections	24051	24179	24226	24481
Redundancy	6.2	6.2	6.2	6.3
Completeness (%)	96.8	97.2	97.3	98.4
Signal ($\langle I \rangle / \sigma \langle I \rangle$) ^a	21.5 (2.3)	21.0 (2.1)	20.9 (2.0)	20.4 (1.9)
R _{sym} (%)	8.1	8.7	9.1	8.8
Overall figure of merit	0.71			
MAD Structure Factor Ratios^b and Anomalous Scattering Factors^c				
Wavelength (λ)	0.9879	0.9783	0.9790	0.9611
0.9879	0.033	0.040	0.032	0.026
0.9793		0.047	0.029	0.044
0.9790			0.063	0.036
0.9611				0.050
f' (e)	-4.87	-9.96	-8.06	-4.15
f'' (e)	0.47	3.77	6.28	4.12
Refinement Statistics				
R _{cryst} (%)	25.3			
R _{free} (%)	28.4			
Average B (\AA^2)	24.8 protein/31.7 solvent			
No. of water molecules	88			
RMSD bond lengths (\AA)	0.0126			
RMSD bond angles ($^\circ$)	1.745			
RMSD B values (\AA^2)	0.837/1.487 bonds/angles main chain 1.021/1.594 bonds/angles side chains			

^aValues in parentheses are for the highest resolution shell (1.73-1.70 \AA).

$R_{\text{sym}} = 100 \times \sum |I - \langle I \rangle| / \sum I$ where I is the integrated intensity of a given reflection.

^b $\text{RMS}(\Delta |F|) / \text{RMS}(|F|)$ where $\Delta |F|$ is the Bijvoet difference at one wavelength (values on the diagonal) or the dispersive differences between two wavelengths (values off the diagonal).

- 5 ^cAnomalous components of the Se scattering factors as a function of wavelength as determined by SOLVE (Terwilliger and Eisenberg, 1983).

^dAll rounds of refinement included data for which $|F| > 2.0\sigma$. $R \text{ value} = \sum |F_p(\text{obs}) - F_p(\text{calc})| / \sum F_p(\text{obs})$, where F_p is the structure factor amplitude. The free R value was calculated from 10% of the data that was excluded from the refinement (Brünger,

- 10 1992).

Amino Acid Residues and the Homer Binding Domain

Mutation	Expression Level (Western Blot)	Binding ^a
Homer 2 EVH WT	++	+ -
F7A	-	ND
F7R	+	+
S8L	+	-
N23A	++	+
S28A	+	-
V34M	++	+
S35V	++	+
D39A	++	-
R42E	++	-
R42A	++	+
R46A	++	-
R46C	++	+
I48A	++	+
N58A	++	+
N64G	++	+
F67S	+	-
K69A	++	+
Q72A	++	+
F74A	++	+
F74L	++	+
F90S	++	+
E93K	+	+

WO 00/11204

PCT/US99/18973

H95A	++	+
L96S	+	+
F109C	++	+

^a (-) indicates substantially reduced binding relative to wild-type (+).

Table 2. WASP EVH1 Mutations**Table 2A - β 1 region**

WASP Residue/Mutation	Homer Residue
Exposed	
L39M	Met 1
C43W	Pro 5
L46P	Ser 8
T48I	Arg 10
E133K	His 95
Buried/partially buried	
T45M	Phe 7
A47D	Thr 9
A49E	Ala 11

Table 2B - β 3 region

WASP Residue/Mutation	Homer Residue
Exposed	
S82P/F	Arg 42
Buried/partially buried	
F84L	Val 44
R86C/H/P/L	Arg 46
G89D	Ser 49

Table 2C – Other mutations

WASP Residue/Mutation	Homer Residue
Exposed	
P58L	Pro 18
E131K	Glu 93
Buried/partially buried	
H68P	Ser 28
V75M	Ser 35
Y107S/C	Phe 67

WO 00/11204

PCT/US99/18973

G125R	Gly 87
F128S	Phe 90
A134T/V	Leu 96

Other

A56V

W97C

-

-

Homer Sequence Listing

SEQ ID No.	Sequence
1	Human Homer 1a (nucleic acid)
2	Human Homer 1a (amino acid)
3	Human Homer 1b (nucleic acid)
4	Human Homer 1b (amino acid)
5	Homer 1c (nucleic acid)
6	Homer 1c (amino acid)
7	Human Homer 2a (nucleic acid)
8	Human Homer 2a (amino acid)
9	Human Homer 2b (nucleic acid)
10	Human Homer 2b (amino acid)
11	Human Homer 3 (nucleic acid)
12	Human Homer 3 (amino acid)
13	peptide binding—core region: PPXXFR
14	peptide binding—extended region: ALTPPSPFRD
15	Homer interacting protein: rat I30 (nucleic acid)
16	Homer interacting protein: rat I30 (amino acid)
17	Homer interacting protein: rat I42 (nucleic acid)
18	Homer interacting protein: rat I42 (amino acid)
19	Homer interacting protein: human I30 (nucleic acid)
20	Homer interacting protein: human I30 (amino acid)
21	Homer interacting protein: human I42 (nucleic acid)
22	Homer interactin protein: human I42 (amino acid)
23	Mouse Homer 1a (nucleic acid)
24	Mouse Homer 1a (amino acid)
25	Mouse Homer 1b (nucleic acid)
26	Mouse Homer 1b (amino acid)
27	Mouse Homer 2a (nucleic acid)

28	Mouse Homer 2a (amino acid)
29	Mouse Homer 2b (nucleic acid)
30	Mouse Homer 2b (amino acid)
31	Mouse Homer 3 (nucleic acid)
32	Mouse Homer 3 (amino acid)
33	Rat Homer 1a (nucleic acid)
34	Rat Homer 1a (amino acid)
35	Rat Homer 1b (nucleic acid)
36	Rat Homer 1b (amino acid)
37	Rat Homer 1c (nucleic acid)
38	Rat Homer 1c (amino acid)
39	Rat Shank 3a (nucleic acid)
40	Rat Shank 3a (amino acid)
41	Human Homer 3a (nucleic acid)
42	Human Homer 3a (amino acid)
43	Rat INADL partial nucleic acid sequence
44	Rat INADL partial amino acid sequence

What is claimed is:

1. A method for identifying a compound that modulates a cellular response mediated by a cell-surface receptor comprising:
 - (a) incubating the compound and a cell expressing a cell-surface receptor and a Homer protein under conditions sufficient to permit the compound to interact with the cell;
 - (b) exposing the cell to a cell-surface receptor ligand; and
 - (c) comparing a cellular response to said ligand in a cell incubated with said compound with the response of a cell not incubated with said compound, thereby identifying a compound that modulates a cellular response.
2. The method of claim 1, wherein said cell-surface receptor is a glutamate receptor.
3. The method of claim 2, wherein the glutamate receptor is a Group I metabotropic glutamate receptor or ionotropic glutamate receptor.
4. The method of claim 3, wherein the metabotropic glutamate receptor is selected from the group consisting of metabotropic glutamate receptor 1a and metabotropic glutamate receptor 5.
5. The method of claim 3, wherein the ionotropic glutamate receptor is selected from the group consisting of NMDA glutamate receptor of the class NR2B and NMDA glutamate receptor of the class NR2D.

6. The method of claim 1, wherein said Homer protein is selected from the group consisting of Homer 1a, Homer 1b, Homer 1c, Homer 2a, Homer 2b and Homer 3.

7. The method of claim 1, wherein said Homer protein is Homer 1a.

8. The method of claim 1, wherein said cellular response is an increase or decrease in calcium mobilization.

9. The method of claim 1, wherein said compound is selected from peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds, biological agents, antibodies, neurotropic agents, combinatorial compound libraries and anti-epileptic agents.

10. The method of claim 1, wherein said cell is selected from the group consisting of a neuronal cell, a glial cell, a cardiac cell, a bronchial cell, a uterine cell, a testicular cell, a liver cell, a renal cell, an intestinal cell, a thymus cell, a spleen cell, a placental cell, a skeletal muscle cell and a smooth muscle cell.

11. A method for identifying a compound that modulates a cellular response mediated by an intracellular protein comprising:
- (a) incubating the compound and a cell expressing an intracellular protein and a Homer protein under conditions sufficient to permit the compound to interact with the cell;
 - (b) exposing the cell to conditions that activate said intracellular protein; and
 - (c) comparing a cellular response in a cell incubated with said compound with the cellular response of a cell not incubated with said compound, thereby identifying a compound that modulates a cellular response.
12. The method of claim 11, wherein said intracellular protein is selected from the group consisting of an inositol trisphosphate receptor, a ryanodine receptor, I42, I30, hInaD, and ACK-2.
13. The method of claim 11 wherein said Homer protein is selected from the group consisting of Homer 1a, Homer 1b, Homer 1c, Homer 2a, Homer 2b and Homer 3.
14. The method of claim 11, wherein said Homer protein is Homer 1a.
15. The method of claim 11, wherein said cellular response is an increase or decrease in calcium mobilization.
16. The method of claim 11, wherein the compound is selected from peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds, biological agents, antibodies, neurotropic agents and anti-epileptic agents.

17. A method for identifying a compound that modulates receptor-activated calcium mobilization comprising:
- (a) incubating the compound and a cell expressing a Homer protein under conditions sufficient to permit the compound to interact with the cell;
 - (b) exposing the cell to conditions sufficient to activate calcium mobilization; and
 - (c) comparing the cellular response in a cell incubated with said compound with the cellular response of a cell not incubated with said compound, thereby identifying a compound that modulates receptor-activated calcium mobilization.
18. The method of claim 17, wherein said Homer protein is selected from Homer 1a, Homer 1b, Homer 1c, Homer 2a, Homer 2b, Homer 3.
19. The method of claim 17, wherein said Homer protein is Homer 1a.
20. The method of claim 17, wherein said cell response is an increase in calcium mobilization.
21. The method of claim 17, wherein said cell response is a decrease in calcium mobilization.
22. The method of claim 17, wherein the compound is selected from peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds, biological agents, antibodies, neurotropic agents and anti-epileptic agents.

23. A method of modulating receptor-mediated calcium mobilization comprising exposing a cell to a compound in a sufficient amount to modulate the calcium mobilization that normally occurs when a cell is exposed to an amount of ligand sufficient to activate an intracellular signaling pathway.

24. The method of claim 23, wherein the compound is selected from peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds, biological agents, antibodies, neurotropic agents and anti-epileptic agents.

25. The method of claim 23, wherein the ligand is an agonist or antagonist of metabotropic glutamate receptors.

26. The method of claim 23, wherein the intracellular signaling pathway is an inositol trisphosphate signaling pathway.

27. A method of identifying a compound that inhibits Homer protein activity comprising:

- (a) designing a potential inhibitor for Homer protein activity that will form non-covalent bonds with amino acids in a Homer binding site based upon the crystal structure co-ordinates of Homer protein binding domain;
- (b) synthesizing the inhibitor; and
- (c) determining whether the inhibitor inhibits Homer protein activity.

28. The method of claim 27, wherein the crystal structure coordinates of the Homer protein binding domain are obtained from a Homer protein crystal having orthorhombic space group symmetry $P2_12_12_1$ with $a = 33.79$, $b = 51.40$, and $c = 66.30$ Angstroms.

29. The method of claim 28, wherein the coordinates of the Homer protein binding domain are obtained by means of computational analysis.

30. The method of claim 27, wherein the potential inhibitor is designed to form hydrogen bonds with tryptophan²⁴, phenylalanine⁷⁴, threonine⁶⁶, threonine⁶⁸, glutamine⁷⁶, alanine⁷⁸, threonine⁷⁰, and valine⁸⁵ of the Homer binding domain.

31. A method for identifying a compound that affects the formation of cell surface receptors into clusters, comprising:

- (a) incubating the compound and a cell expressing a Homer protein and a Shank protein under conditions sufficient to allow the compound to interact with the cell;
- (b) determining the effect of the compound on the formation of cell-surface receptors into clusters; and
- (c) comparing the formation of cell-surface receptors into clusters of said cell contacted with said compound with the formation of cell-surface receptors into clusters in a cell not contacted with said compounds, thereby identifying a compound that affects the formation of cell-surface receptors into clusters.

32. The method of claim 31, wherein the cell-surface receptor is a NMDA receptor.

33. The method of claim 31, wherein the cell-surface receptor is a metabotropic glutamate receptor.

34. The method of claim 33, wherein the metabotropic glutamate receptor is a group I metabotropic glutamate receptor.

35. The method of claim 34, wherein the metabotropic glutamate receptor is metabotropic glutamate receptor 1 α .

36. The method of claim 34, wherein the metabotropic glutamate receptor is metabotropic glutamate receptor 5.

37. The method of claim 31, wherein said Homer protein is selected from the group consisting of Homer 1a, Homer 1b, Homer 1c, Homer 2a, Homer 2b and Homer 3.

38. The method of claim 31, wherein the compound is selected from peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds, biological agents, antibodies, neurotropic agents and anti-epileptic agents.

39. The method of claim 31, wherein said Shank protein is selected from the group consisting of Shank 1a, Shank 1b and Shank 3, and cortactin binding protein.

40. The method of claim 31, wherein the effect is inhibition of the recruitment of cell-surface receptors into clusters.

41. The method of claim 31, wherein the effect is stimulation of the formation of cell-surface receptors into clusters.

42. The method of claim 31, wherein said cell is selected from the group consisting of a neuronal cell, a glial cell, a cardiac cell, a bronchial cell, a uterine cell, a testicular cell, a liver cell, a renal cell, an intestinal cell, a thymus cell, a spleen cell, a placental cell, a skeletal muscle cell and a smooth muscle cell.

43. The method of claim 31, wherein said compound is selected from peptides, peptidomimetics, polypeptides, pharmaceuticals, chemical compounds, biological agents, antibodies, neurotropic agents and anti-epileptic agents.

44. A method of treating a disorder associated with glutamate receptors comprising administering to a subject in need thereof a therapeutically effective amount of a compound that modulates Homer protein activity.

45. The method of claim 44, wherein said Homer protein is selected from the group consisting of Homer 1a, Homer 1b, Homer 1c, Homer 2a, Homer 2b, and Homer 3.

46. The method of claim 44, wherein said Homer protein is Homer 1a.

47. The method of claim 44, wherein said disorder is selected from epilepsy, glutamate toxicity, disorders of memory, disorders of learning, stroke, schizophrenia, Alzheimer's disease, tissue degeneration and disorders of brain development.

48. A method of treating a disorder associated with Homer protein activity comprising administering to a subject in need thereof a therapeutically effective amount of a compound that modulates Homer protein activity.

49. The method of claim 48, wherein said disorder is a cardiac disorder, a disorder of musculature, a vasculature disorder, a neurological disorder, a psychiatric disorder, a renal disorder, a uterine disorder or a disorder of bronchial tissue.

50. An isolated nucleic acid encoding Homer protein 1b, wherein said nucleic acid has the nucleotide sequence set forth in SEQ. I.D NO: 3.

51. An isolated Homer protein 1b, wherein said protein has substantially the same amino acid sequence as set forth in SEQ ID NO:4.

52. An isolated nucleic acid encoding Homer protein 1c, wherein said nucleic acid has the nucleotide sequence set forth in SEQ ID NO: 5.

53. An isolated Homer protein 1c, wherein said protein has substantially the same amino acid sequence as set forth in SEQ ID NO:6.

54. An isolated nucleic acid encoding Homer protein 2a, wherein said nucleic acid has the nucleotide sequence set forth in SEQ ID No: 7.

55. An isolated Homer protein 2a, wherein said protein has substantially the same amino acid sequence as set forth in SEQ ID NO:8.

56. An isolated nucleic acid encoding Homer protein 2b, wherein said nucleic acid has the nucleotide sequence set forth in SEQ ID NO: 9.

57. An isolated Homer protein 2b, wherein said protein has substantially the same amino acid sequence as set forth in SEQ ID NO:10.

58. An isolated nucleic acid encoding Homer protein 3, wherein said nucleic acid has the nucleotide sequence set forth in SEQ ID NO: 11.
59. An isolated Homer protein 3, wherein said protein has substantially the same amino acid sequence as set forth in SEQ ID NO:12.
60. An isolated peptide, wherein said peptide is set forth in SEQ ID NO:13, wherein R can be arginine, or zero, or one other amino acid residue.
61. An isolated peptide, wherein said peptide is set forth in SEQ ID NO:14.
62. An isolated nucleic acid encoding a Homer Interacting Protein, wherein said nucleic acid has the nucleotide sequence set forth in SEQ ID No:15 or SEQ. ID No: 17.
63. An isolated Homer Interacting Protein, wherein said protein has substantially the same amino acid sequence as set forth in SEQ ID NO: 16 or 18.
64. An isolated nucleic acid encoding Homer Interacting Protein, wherein said nucleic acid is encoded by SEQ ID NO: 19.
65. An isolated Homer Interacting Protein, wherein said protein has substantially the same amino acid sequence as set forth in SEQ ID NO: 20.

66. A substantially purified polypeptide containing a proline-rich region that binds to a polypeptide of the Homer family.
67. The polypeptide of claim 66, wherein said polypeptide is a cell-surface receptor.
68. The polypeptide of claim 66, wherein said polypeptide is an intracellular receptor.
69. A transgenic non-human animal having a transgene that expresses Homer protein 1a chromosomally integrated into the germ cells of the animal.
70. The transgenic animal of claim 69, wherein the animal is murine.
71. A method for affecting the natural aging process comprising administering to a subject in need thereof, an effective amount of a compound that modulates Homer protein activity.

Figure 1

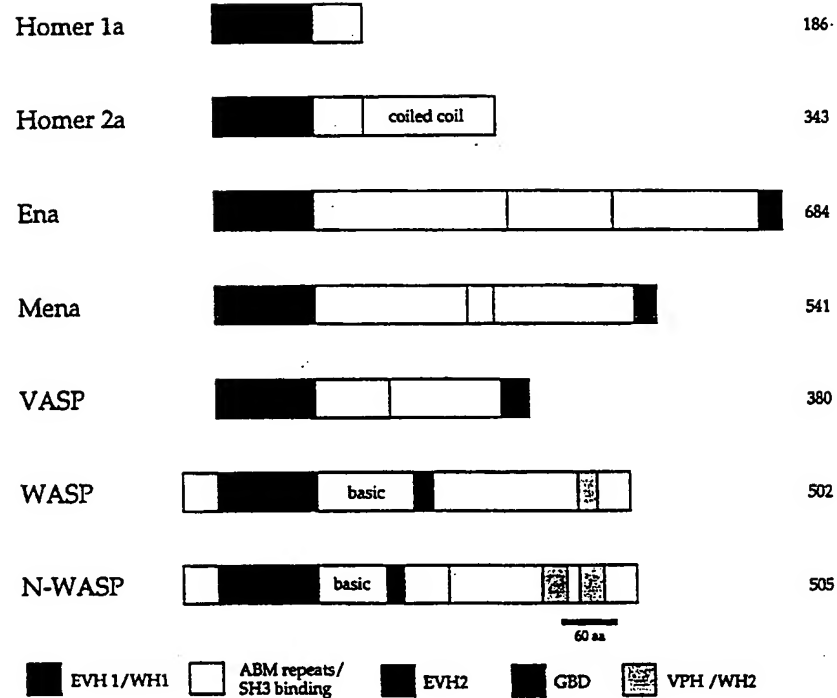


Figure 2

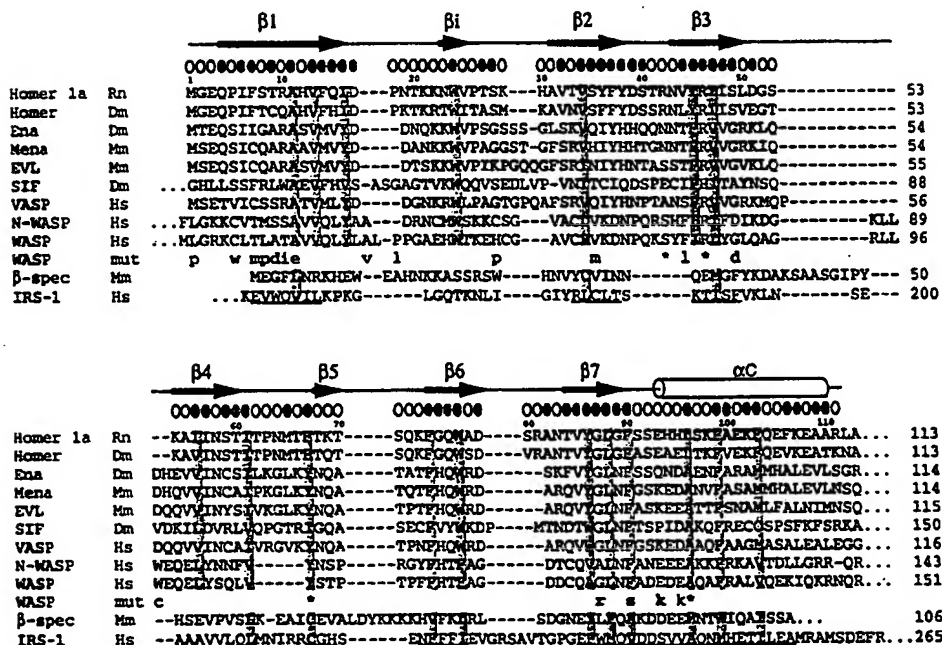


Figure 3

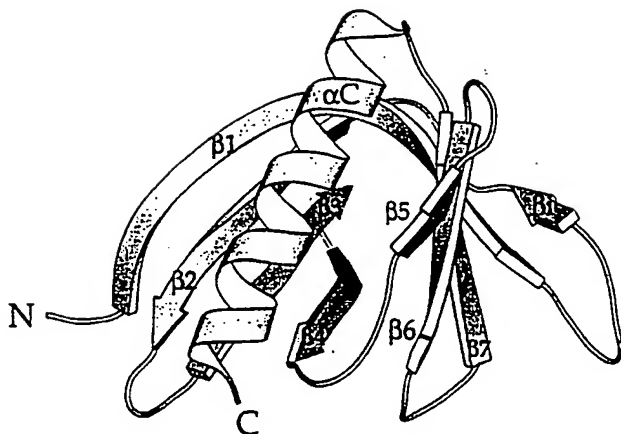


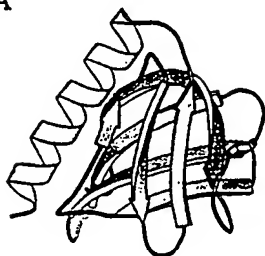
Figure 4

EVH1 Domain (Homer)

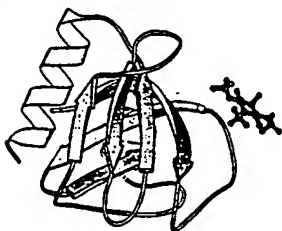
PH Domain (Spectrin)

PTB Domain (IRS-1)

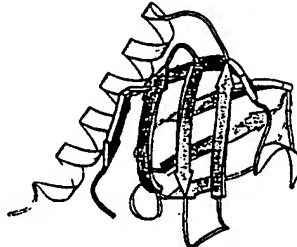
A



B



C



D



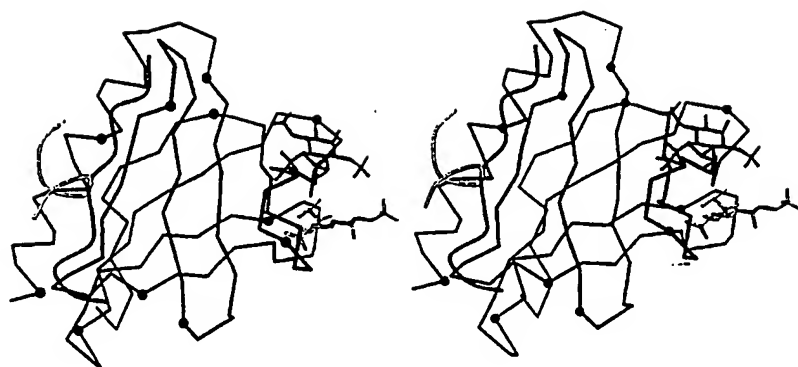
E

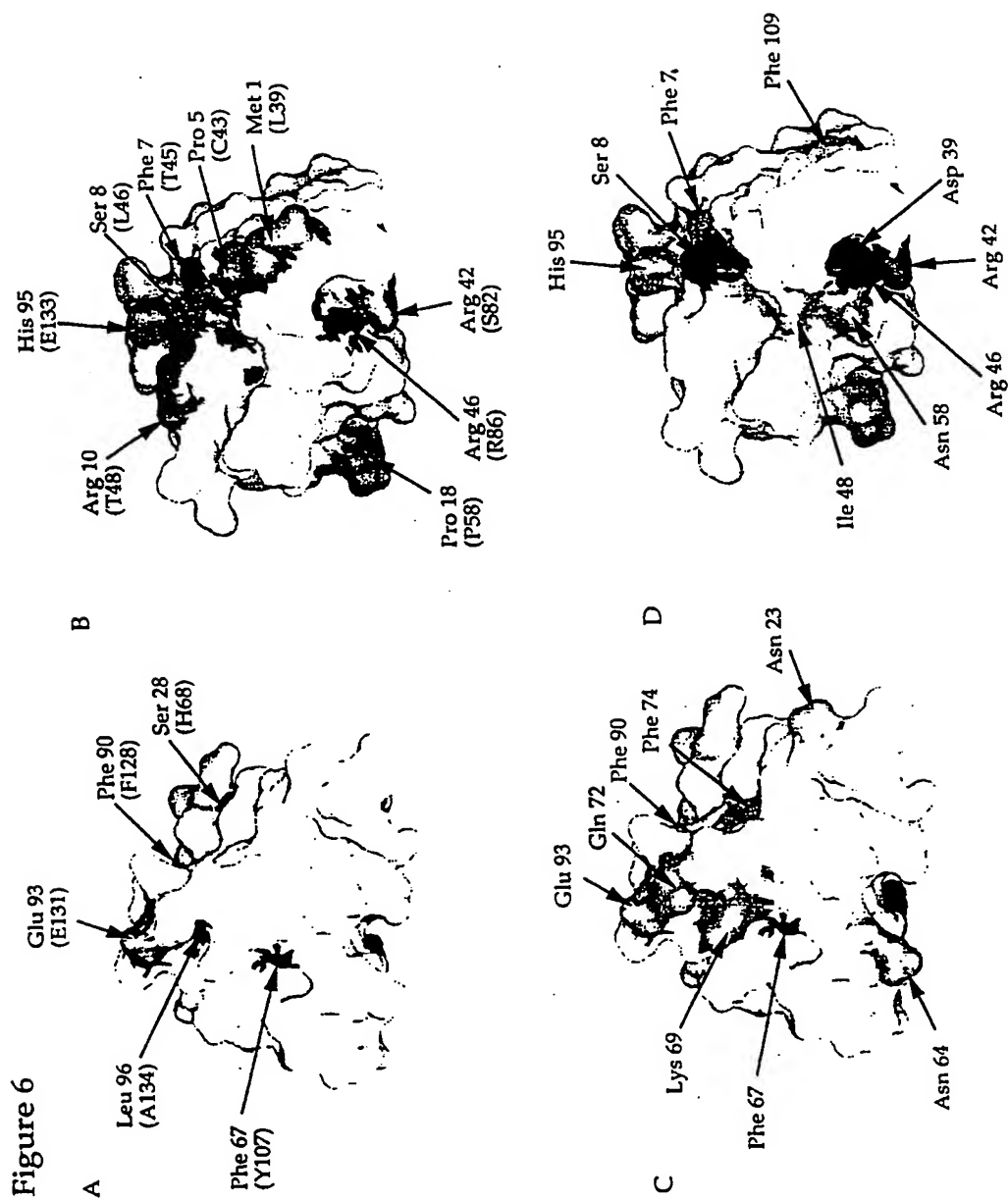


F



Figure 5





H-Homer-1a

MGEQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAIINSTITP
NMTFTKTSQKFGQWADSRANTVYGLGFSSEHLSKFAEKFOEFKEAARLAKEKSQEKME
LTSTPSQESAGGDLQSPLTPESINGTDDERTPDVTQNSEPRAEPTQNALPFSHSSAISKHWEA
ELATLKGNNAKLTAALLESTANVKQWKQQLAAYQEEAERLHKRVISGLMSIGI

Figure 7

H-Homer-1bGenBank

ATGGGGGAGCAGCCGATTTTCAGCACTCGAGCTCATGTCTTCCAAATTGACCCAAACA
CAAAGAAGAACTGGGTACCCACCAGCAAGCATGCAGTTACTGTGTCTTATTTCTATGA
CAGCACAAAGAAATGTGTATAGGATAATCAGTTTAGATGGCTCAAAGGCAATAATAAAT
AGTACCATCACCCCAAACATGACATTTACTAAAACATCTCAGAAAGTTTGGCCAGTGGG
CTGATAGCCGGGCAAACACCGTTTATGGATTGGGATTCTCCTCTGAGCATCATCTTTCG
AAATTTGCAGAAAAGTTTCAGGAATTTAAAGAAGCTGCTCGACTAGCAAAGGAAAAA
TCACAAGAGAAGATGGAACCTTACCAGTACACCTTCACAGGAATCCGCAGGCGGGGAT
CTTCAGTCTCCTTTAACACCGGAAAGTATCAACGGGACAGATGATGAAAGAACACCTG
ATGTGACACAGAACTCAGAGCCAAGGGCTGAACCAACTCAGAATGCATTGCCATTTTC
ACATAGTTCAGCAATCAGCAAACATTGGGAGGCTGAACTGGCTACCCTCAAAGGAAAT
AATGCCAAACTCACTGCAGCCCTGCTGGAGTCCACTGCCAATGTGAAACAATGGAAAC
AGCAACTTGCTGCCTATCAAGAGGAAGCAGAACGTCTGCACAAGCGGGTGACTGAACT
TGAATGTGTTAGTAGCCAAGCAAATGCAGTACATACTCATAAGACAGAATTAAATCAG
ACAATACAAGAACTGGAAGAGACACTGAACTGAAGGAAGAGGAAATAGAAAGGTT
AAAACAAGAAATTGATAATGCCAGAGAACTACAAGAACAGAGGGATTCTTTGACTCA
GAAACTACAGGAAGTAGAAATTCGGAACAAAGACCTGGAGGGACAACCTGTCTGACTT
AGAGCAACGTCTGGAGAAAAGTCAGAATGAACAAGAAGCTTTTCGCAATAACCTGAA
GACACTCTTAGAAATTCTGGATGGAAAAGATATTTGAACTAACAGAATTACGAGATAAC
TTGGCCAAGCTACTAGAATGCAGCTAAGGAAAGTGAAATTCAGTGCCAATTAATTAA
AAGATACACTGTCTCTCTTCATAGGACTGTTTAGCTCTGCATCAAGATTGCACAAAAAA
AAAAAAAAAAAA

Figure 8

H-Homer-1b

MGEQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAIINSTITP
NMTFTKTSQKFGQWADSRANTVYGLGFSSEHLSKFAEKQEFKEAARLAKEKSQEKME
LTSTPSQESAGGDLQSPLTPESINGTDDERTPDVTQNSEPRAEPTQNALPFSHSSAISKHWEA
ELATLKGNNAKLTAALLESTANVKQWKQQLAAYQEEAERLHKRVTELECVSSQANAVHT
HKTELNQTIQEEETLKLKEEEIERLKQEIDNARELQEQRDSLTKLQEVERNKDLEGQLS
DLEQRLEKSQNEQEAFRNNLKTLEILDGKIFELTEL RDNLAKLLECS*

Figure 9

H-Homer-2aGenBank

ATGGGGGAGCAGCCGATCTTCACCACCCGAGCGCATGTCTTCCAGATTGACCCCAACA
CCAAGAAGAACTGGATGCCTGCGAGCAAGCAGGCGGTACCGTTTCCTACTTCTATGA
TGTCACAAGGAACAGCTATCGGATCATCAGTGTGGACGGAGCCAAGGTGATCATAAAC
AGCACAATCACACCGAATATGACCTTCACCAAAACGTACAGAAAGTTTGGGCAGTGGG
CCGACAGCAGAGCCAACACAGTGTGGTTGGGGTTTTCTCTGAGCAGCAGCTGAC
AAAGTTTGCAGAGAAATTCCAGGAGGTGAAAGAAGCTGCCAAGATAGCCAAAGACAA
GACGCAGGAGAAAATCGAGACCTCAAGTAATCATTCCCAAGCATCCAGTGTCAACGG
GACGGACGAGGAAAAGGCCTCTCACGCCGGTCCAGCCAACACACAACCTGAAGTCTGA
GAATGACAAGCTGAAGATTGCCTTGACGCAGAGCGCAGCCAACGTGAAGAAGTGGGA
GATCGAGCTGCAGACCTTTCGGGAGAGCAATGCACGGCTGACCACAGCACTGCAGGA
GTCGGCAGCCAGTGTGGAGCAGTGAAGAGGCAGTTCTCCATCTGCCGTGATGAGAAT
GACCGGCTCCGCAACAAGATTGATGAGCTGGAAGAACAATGCAGTGAGATCAACAGA
GAGAAGGAGAAGAACACGCAGCTGAAGAGGAGGATCGAGGAGCTGGAGGCAGAGCT
CCGAGAAAAGGAGACAGAGCTGAAAGATCTCCGAAAACAAAGTGAAATCATACCTCA
GCTCATGTCAGAGTGCGAATATGTCTCTGAGAAGCTAGAGGCGGCAGAGAGAGACAA
TCAAAACCTGGAAGACAAAGTGCGTTCTTAAAGACAGACATTGAGGAGAGCAAATA
CCGACAGCGCCACCTGAAGGTGGAGTTGAAGAGCTTCCTGGAGGTGCTGGACGGGAA
GATTGACGACCTGCATGACTTCCGCCGAGGGCTCTCCAAGCTGGGCACCGATAACTAG
GGCTGGCCGAGGCCAGGCCCGCCCGTGAGTCCAAGCGTGTGTGCGAGACCAGATA
GCTCTAGGACGTTCTTCTGTGTGCATTGCTTCTGTAAATGCAGGCGCAGTTTGTCTGT
TTCCAAACCAGTTGTGCCGTCCACTCACTCTTTTCAGAATAGAAATCTCCTCTCGCTT
CTCTGGCCTTGTGAGGTTGTGGACAACTGGAAGATTCTGACTCAGGAATCCAGAACTA
GGTCTACCTTCAACATTTATGCAGTCAGGGCAGGGATGTTTATATCTTTCATAAGGGCT
GTTGCAACCATATGAACTGAAAAACACGCATTTTGTAAATCCAAATATTGATATTCTTT
ACACCAAGCCATCAGGCTCCTTTTATCAAATAGCATTCAAGATTTTGAATGTCCACCA
GACACCAGCCCCGGGGGGCACAGAGAGAACAACATTCCTCTCTGTCAACATCGAGAG
GCTTTAAAACAACTGTTTAGTGGAACCTTTCTGAGAGATGGAAAACAAGCTTCTGGTG
GGTGCATTTTCTGGCCCGGAGTTGCCTGCATCCACGCTACTGCCCCCTGCCCCCGCCC
CCCCAGTTTGTACGGTTGCAACAGTGTTCTTTTCTTGGTTTTAATTTCTGAGCAGATGA
TTTGCTGTGGGAACAGCACACAGTGAGGGTGCCTAGCACAAATGTCTGGCACAAAGTAG
GTGCTTAATAAATATTTGTTCAATTAATAAAAAA

Figure 10

H-Homer-2a

MGEQPIFTTRAHV FQIDPNTKKNWMPASKQAVTVSYFYDVTRNSYRIISVDGAKVIINSTIT
PNMTFTKTSQKFGQWADSRANTVFGLGFSSEQQLTKFAEKFEVKEAAKIAKDKTQEKIE
TSSNHSQASSVNGTDEEKASHAGPANTQLKSENDKLKIALTQSAANVKKWEIELQTLRESN
ARLTTALQESAASVEQWKRQFSICRDENDRLRNKIDELEEQCSEINREKEKNTQLKRRIEEL
EAELEKETELKDLRKQSEIIPQLMSECEYVSEKLEAAERDNQNLEDKVRSLKTDIEESKYR
QRHLKVELKSFLEVLDGKIDDLHDFRRGLSKLGTDN*

Figure 11

H-Homer-2bGenBank

ATGGGGGAGCAGCCGATCTTCACCACCCGAGCGCATGTCTTCCAGATTGACCCCAACA
CCAAGAAGAACTGGATGCCTGCGAGCAAGCAGGCGGTACCGTTTCCTACTTCTATGA
TGTCACAAGGAACAGCTATCGGATCATCAGTGTGGACGGAGCCAAGGTGATCATAAAC
AGCACAATCACACCGAATATGACCTTCACCAAAACGTACAGAAAGTTTGGGCAGTGGG
CCGACAGCAGAGCCAACACAGTGTGGTGGGGTTTTCTCTGAGCAGCAGCTGAC
AAAGTTTGCAGAGAAATTCAGGAGGTGAAAGAAGCTGCCAAGATAGCCAAAGACAA
GACGCAGGAGAAAATCGAGACCTCAAGTAATCATTCCCAAGAATCTGGGCGTGAAAC
CCCATCTTCTACTCAGGCATCCAGTGTCAACGGGACGGACGAGGAAAAGGCCTCTCAC
GCCGGTCCAGCCAACACACAAGTGAAGTCTGAGAATGACAAGCTGAAGATTGCCTTGA
CGCAGAGCGCAGCCAACGTGAAGAAGTGGGAGATCGAGCTGCAGACCTTCGGGAGA
GCAATGCACGGCTGACCACAGCACTGCAGGAGTCGGCAGCCAGTGTGGAGCAGTGGG
AGAGGCAGTTCTCCATCTGCCGTGATGAGAATGACCGGCTCCGCAACAAGATTGATGA
GCTGGAAGAACAATGCAGTGAGATCAACAGAGAGAAGGAGAAGAACACGCAGCTGA
AGAGGAGGATCGAGGAGCTGGAGGCAGAGCTCCGAGAAAAGGAGACAGAGCTGAAA
GATCTCCGAAAACAAAGTGAATCATACCTCAGCTCATGTGAGAGTGCGAATATGTCT
CTGAGAAGCTAGAGGCGGCAGAGAGAGACAATCAAAACCTGGAAGACAAAGTGCGTT
CCTTAAAGACAGACATTGAGGAGAGCAAATACCGACAGCGCCACCTGAAGGTGGAGT
TGAAGAGCTTCCTGGAGGTGCTGGACGGGAAGATTGACGACCTGCATGACTTCCGCCG
AGGGCTCTCCAAGCTGGGCACCGATAACTAGGGCTGGCCGAGGCCAGGCCCGGCCG
TGAGTCCCAAGCGTGTGTGCGAGACCAGATAGCTCTAGGACGTTCTTCTGTGTGCATTG
CTTCTGTAAATGCAGGCGCAGTTTGTCTGTGTTTCCAAACCAGTTGTGCCGTCCACTCAC
TCCTTTTCAGAATAGAAATCTCCTCTCGCTTCTCTGGCCTTGTGAGGTTGTGGACAACT
GGAAGATTCTGACTCAGGAATCCAGAACTAGGTCTACCTTCAACATTTATGCAGTCAG
GGCAGGGATGTTTATATCTTTCATAAGGGCTGTTGCAACCATATGAACTGAAAAACA
CGCATTTTGTAAATCCAAATATTGATATTCTTTACACCAAGCCATCAGGCTCCTTTTATC
AAATAGCATTACAGAGTATTTGAATGTCCACCAGACACCAGCCCCGGGGGGCACAGAG
AGAACAACATTCTCTGTCAACATCGAGAGGCTTTAAACAACCTGTTAGTGGA
CTTTCTGAGAGATGGAAAACAAGCTTCTGGTGGGTGCATTTCTGGCCCGGAGTTGCCT
GCATCCACGCTACTGCCCCCTGCCCCCGCCCCCAGTTTGTACGGTTGCAACAGTGT
TCCTTTTCTTGGTTTTAATTTCTGAGCAGATGATTTGCTGTGGGAACAGCACACAGTGA
GGGTGCCTAGCACAATGTCTGGCACAAGTAGGTGCTTAATAAATATTTGTTCAATTA
AAAAAA

Figure 12

H-Homer-2b

MGEQPIFTTRAHV FQIDPNTKKNWMPASKQAVTVSYFYDVTRNSYRIISVDGAKVIINSTIT
PNMTFTKTSQKFGQWADSRANTVFGLGFSSEQQLTKFAEKFQEVKEAAKIAKDKTQEKIE
TSSNHSQESGRETPSSTQASSVNGTDEEKASHAGPANTQLKSENDKLIKIALTQSAANVKK
WEIELQTLRESNARLTTALQESAASVEQWKRFQFICRDENDRLRNKIDELEEQCSEINREKE
KNTQLKRRIEELEAELREKETELKDLRKQSEIHPQLMSECEYVSEKLEAAERDNQNLEDKV
RSLKTDIEESKYRQRHLKVELKSFLEVLDGKIDDLHDFRRGLSKLGTDN*

Figure 13

H-Homer-3GenBank

GCACGAGGGCGCATGACTAGTTGGGGCCAAACCAGTGCTCCTGCCACCTCTCTGGCTG
CCCCCTAGAGCCTGCCCATCCCAGCCTGACCAATGTCCACAGCCAGGGAGCAGCCAAT
CTTCAGCACACGGGCGCACGTGTTCCAAATTGACCCAGCCACCAAGCGAAACTGGATC
CCAGCGGGCAAGCACGCACTCACTGTCTCCTATTTCTACGATGCCACCCGCAATGTGTA
CCGCATCATCAGCATCGGAGGCGCCAAGGCCATCATCAACAGCACTGTCACTCCCAAC
ATGACCTTCACCAAAACTTCCCAGAAGTTCGGGCAGTGGGCCGACAGTCGCGCCAACA
CAGTCTACGGCCTGGGCTTTGCCTCTGAACAGCATCTGACACAGTTTGCCGAGAAGTTC
CAGGAAGTGAAGGAAGCAGCCAGGCTGGCCAGGGAGAAATCTCAGGATGGCGGGGA
GCTCACCAGTCCAGCCCTGGGGCTCGCTCCCACCAGGTCCCCCGAGCCCTCTCGTCA
GTGCCAACGGCCCCGGCGAGGAAAACTGTTCCGCAGCCAGAGCGCTGATGCCCCCG
GCCCCACAGAGCGCGAGCGGCTAAAGAAGATGTTGTCTGAGGGCTCCGTGGGCGAGG
TACAGTGGGAGGCCGAGTTTTTCGCACTGCAGGACAGCAACAACAAGCTGGCAGGCG
CCCTGCGAGAGGCCAACGCCGCCGCAGCCAGTGGAGGCAGCAGCTGGAGGCTCAGC
GTGCAGAGGCCGAGCGGCTGCGGCAGCGGGTGGCTGAGCTGGAGGCTCAGGCAGCTT
CAGAGGTGACCCCCACCGGTGAGAAGGAGGGGCTGGGCCAGGGCCAGTCGCTGGAAC
AGCTGGAAGCTCTGGTGCAAACCAAGGACCAGGAGATTCAGACCCTGAAGAGTCAGA
CTGGGGGGCCCCGCGAGGCCCTGGAGGCTGCCGAGCGTGAGGAGACTCAGCAGAAGG
TGCAGACCCGCAATGCGGAGTTGGAGCACCAGCTGCGGGCGATGGAGCGCAGCCTGG
AGGAGGCACGGGCAGAGCGGGAGCGGGCGCGGGCTGAGGTGGGCCGGGCAGCGCAG
CTGCTGGACGTACGCCTGTTTGAGCTGAGTGAGCTGCGTGAGGGCCTGGCCCGCCTGG
CTGAGGCTGCGCCCTGAGCCGGGGCTGGTTTTCTATGAACGATTCCGGCCTGGGATGC
GGGCCAGGCTGCAGGCGGCATAGTTGGGCCCATTCGTCCTGGAAAGGGACTGGGGGG
TCCCAACTTAGCCCTGGGTGGGCCCGGGCGGGNTGGGCTGGGGTGGGCCCCAGTCGGC
TCTGTTGTTGGCAGCTTTGGGGCTGTTTTGAGCTTCTCATTGTGTAGAATTTCTAGAT
CCCCCGATTACATTTCTAAGCGTGAAAAAAAAAAAAAAAAAAAAA

Figure 14

H-Homer-3

MSTAREQPIFSTRAHVFIQIDPATKRNWIPAGKHALTVSYFYDATRNVYRIISIGGAKAINST
VTPNMTFTKTSQKFGQWADSRANTVYGLGFASEQHLTQFAEKFQEVKEAARLAREKSQD
GGELTSPALGLASHQVPPSPLVSANGPGEEKLFRSQSADAPGPTERERLKKMLSEGSVGEV
QWEAEFFALQDSNNKLAGALREANAAAAQWRQQLEAQRAEAERLRQRVAELEAQAASE
VTPTGEKEGLGQGSLEQLEALVQTKDQEIQTLKSQTGGPREALEAAEREETQQKVQTRN
AELEHQLRAMERSLEEARAERERARA EVGRAAQLLDVSLFELSELREGLARLAEAAP

Figure 15

r-i30

CACGCGTCCGTGGCGGAGCTGCAGCAGCTGCAGCAGTTGCAGGAGTTCGATATCCCCA
CGGGCCGGGAGGCTCTGCGGGGCAACCACAGCGCCCTGCTACGGGTGGCCAACTACTG
TGAGGATAACTACTTGCAGGCCACAGACAAGCGGAAGGCGCTGGAAGAGACGATGGC
TTTACCACCCAGGCCCTGGCCAGTGTAGCCTATCAAGTGGGTAACCTGGCGGGGCAC
ACGCTTCGAATGCTGGATCTACAGGGTGTGCCCCTGCGGCAGGTGGAAGCCAAGATGA
GCACACTGGGCCAGATGGTGAACATGCACCTGGAGAAAGTAGCCAGAAGGGAGATTG
GCACGTTGGCCACTGTCTGTGCGGCTGCCCCCTAGCCAGAAGGTCATCCCTCCTGAGAG
CCTGCCTCCCCTCACTCCCTACTGCAGAAAACCCCTCAACTTTGCCTGCTTGGATGATG
TTGGCCATGGAGTCAAGGACTTGAGCACACAGCTGTCACGGACCGGGACCCTGTCTCG
CAAGAGCATAAAGGCGCCCGCTACACCTGCCTCTGCCACGCTGGGGAGACCACCCCGG
ATCCCTGAGCCGGTGCAGCTCCCAGCGGTGCCAGACGGCAAGCTCTCCGCTGCCTCCT
CTGTGTCTTCCTTGGCCTCCGCAGGCAGTGCAGAAGGTGCCAGTGGGATCCCCCAGTC
CAAGGGACAGGTAGCACCTGCAACCCCGCCTCCTCCACCTATAGCGCCTGTAACCTCA
CCTCCTCCACCATTGCCTGCTGAGATCTTCTTGCTGCCCCCTCCGATGGAGGAGTCCCA
GCCCCCTCCGGAAACAGAGTTGCCCCTGCCTCCTCCTCCGGCTCTACAGGGGGATGAA
CTGGGGCTGCTGCCTCCGCCTCCACCAGGTTTGGACCGGATGAGCCCAGCTGGGTCC
CTGCTGCCTACTTGGAGAAAGTGGTGACGCTGTACCCATACACCCGGCAGAAGGACAA
TGAGCTCTCCTTTTCTGAAGGAACCGTCATCTGTGTCACTCGACGCTACTCAGATGGCT
GGTGTGAGGGTGTGAGCTCAGAGGGCACTGGATTCTTCCCAGGGAACTATGTGGAGCC
CAGCTGCTGACAGCCCAGATCTGTCCCTGCCTCTTTGGTGGGCCTCTTGAGCCCCAAGA
AGCCACCTTCCACTCAAAGCTGGACTAAGGACCTGTCTACCTCTTGGGCTGTGAAGTGT
GTTCAAGTCCACACAGCAGTAGGAAGGGGTATGGGATGGGCTAGAGAGTGGTGGTACT
GAGGACGATTGCTCCAGATGGCAAGAACAAAACAAAACCAAGAAGTTAAGTTT
AAGCACCTTGCCCAGAGGACCCCCTAGCTCATGCACCGATCGCCAGCATTGAATAAAA
CTGTTGACCTCCAGGATTGTT

Figure 16

r-i30(1)

HASVAELQQLQLQEFDIPTGREALRGNHSALLRVANYCEDNYLQATDKRKALEETMAFTTQALA
SVAYQVGNLAGHTLRMLDLQGAALRQVEAKMSTLGQMVNMHLEKVARREIGTLATVVRLPPSQK
VIPPELPLTPYCRKPLNFACLDVGHGVKDLSTQLSRTGTLRKSIAKAPATPASATLGRPPRIPEPV
QLPAVPDGKLSAASSVSSLASAGSAEGASGIPQSKGQVAPATPPPPPIAPVTPPPPLPAEIFLLPPME
ESQPPPETELPLPPPALQGDELGLLPPPPPGFGPDEPSWVPAAYLEKVVTLYPYTRQKDNELSFSEG
TVICVTRRYSDGWCEGVSSEGTGFFPGNYVEPSC*

Figure 17

R-I-42CD

Figure 18a

ATGATGACAAACCGAGATGGACGTGACTACTTCATCAATCACATGACACAGGCAATCC
CATTTGATGACCCTCGGTTTGACAGCTGCCAAATCATTCCCCCAGCTCCACGGAAGGTG
GAGATGAGGAGGGACCCTGTGCTGGGCTTTGGGTTTCGTGGCAGGGAGTGAAAAGCCA
GTGGTCGTTTCGATCGGTAACACCAGGTGGCCCTTCAGAAGGCAAGCTGATCCCGGGAG
ATCAAATTGTAATGATTAATGATGAACCAAGTCAGCGCTGCGCCAAGAGAGAGGGTCAT
CGACCTGGTCAGGAGCTGCAAAGAATCGATTCTGTTCACTGTCATCCAGCCTTATCCTT
CTCCCAAATCAGCATTTATTAGTGCTGCTAAAAAGGCAAGATTGAAGTCCAATCCAGT
CAAAGTACGCTTTTCCGAAGAGGTTCATCATCAATGGTCAGGTGTCGGAAACTGTTAAA
GACAATTCACTTCTTTTATGCCAAATGTTTTGAAAGTCTACTTGGAAAATGGACAGAC
CAAATCCTTTTCGCTTTGACTGCAGCACTTCCATTAAGGATGTCATCTTAAGTCTGCAAG
AGAAGCTGTCTATCAAAGGCATTGAGCACTTCTCTCTCATGCTGGAGCAGAGAACTGA
AGGGGCCCGGCACCAAGCTGCTCTTACTTCATGAACAGGAGACACTCACTCAGGTGACA
CAGAGGCCGAGTTCCCATAGATGAGGTGTCTTTCCGAATCAGTTTTGTTCCCAAGGA
TCCCATGACCTGTTAAGGAGAGATCCAGTTGCTTTCGAGTATCTCTATGTTTCAGAGCT
GTAACGATGTCGTTTCAGGAGCGATTTGGACCAGAGCTGAAATACGACATTGCCTTGCG
GCTGGCCGCTTTACAAATGTACATTGCTACTGTCACCACCAAACAGACGCAGAAAAATC
TCCCTCAAGTACATTGAGAAAGAATGGGGACTAGAGACTTTCTTCCATCTGCTGTACT
TCAGAGCATGAAAGAGAAGAACATCAAGAAAGCGCTCTCCACCTTGTCAAAGCAAA
TCAAAACTTGGTACCACCGGGTAAAAAGCTCTCTGCACTACAAGCTAAGGTCCACTAT
CTCAAGTTCTCTCAGTGACCTGCGACTATACGGGGGCCGTGTGTTCAAGGCAACATTAG
TGCAGGCAGAGAAGCGCTCAGAAGTGACTCTTCTGGTGGGTCCCCGGTATGGCATAAG
CCATGTCATAAACACCAAACCAACCTGGTGGCTCTTTAGCTGACTTCAGCCATGTCA
ACAGGATTGAAATGTTTACTGAAGAGGAGAGTTTGGTGAGGGTGGAGTTGCATGTGCT
CGATGTGAAGCCATTACACTCCTTATGGAGTCATCAGATGCCATGAACCTGGCCTGTC
TGACAGCTGGATACTACCGGTTGCTCGTGGACTCCAGGAGGTCAATATTTAACATGGC
CAACAAGAAAAATGCAGGCACACAGGACACAGGAACGGAATAAAGGCAAGCATA
ATCTCCTTGGTCTGACTGGAACCTGTATGCTTCTAAGCAGAAGGCAGTTGAGATGAC
GGAACAAGAAGCCCAATCACTTATATAGATTCTAAGCAGAAGGCAGTTGAGATGAC
AGACAGCACCTTGTGTCCCAAAGAGCACCGGCACCTTATATATCGACAACACATACAGT
TCAGATGAACCTTAGCCAGCCGCTGACTCAGCCAGGTGATGCACCCTGTGAGGCCGACT
ATAGAAGCCTAGCTCAGCGGTCCCTTTTGACCCTCTCAGGACCAGACACTCTGAAGAA
AGCACAGGAATCTCCGCGAGGAGCTAAAGTGTCTTTATTTTTGGAGATCTTGCCTTAG
ATGATGGCATGAGTCCCCCAACTCTAGGCTATGAAAGAATGTTAGATGAGAATCCAGA
AATGCTGGAGAAGCAGAGGAATCTCTACATCAGCAGTGCCAATGATATGAAAACT
GGACCTCACTCCAGACACAGACAGCATCCAGTTTGTGGCAAATTCAGTATATGCAAC
ATAGGTGATGTGAAGAACTTTGAAGCCCCTGAGGGAATAGAGGAGCCCCTCTTACATG
ACATCTGTTATGCTGAAAACACAGATGATGCAGAAGATGAAGATGAGGTGAGCTGCG
AGGAGGATCTCGTGGTGAGTGAAATCAACCAACCAGCCATCCTTGACCTGTCTGGGTC
AAGTGATGATATTATTGACCTTACAACACTGCCTCCTCCAGAAGGAGATGACAATGAG
GATGACTTCTCCTGCGTTCTCTGAACATGGCCATTGCTGCTCCCCACCTGGTTTTAG
AGACAGTTCTGATGAAGAGGACACTCAGAGCCAGGCAACATCCTTCCATGAGAAACAA
AGAACAAGGCAGCAGCCTGCAGAAATGAGGAGATCCCTGTGTCCCTCATTGATGCTGTG
CCCACAGTGACAGAGGGCAAGTGTGAGAAGGGACTGGACCCTACCGTCGTTTCCACAC
TAGAAGCCCTAGAAGCTCTTTCAGAAGAACAGCAGAAGAGTGAAAATTCAGGTGTAG
CCATCTTGGGGCTTATAGTCCCGAGTCTTCTCAGACTCGGGCAATGAGACTAACTCT
TCTGAAATGACAGAGGGTTCTGAACTAGCTGCAGCACAGAAGCAGTCGGAAAGCCTCT
CCCGCATGTTCTTGGCCACTCATGAAGGTTATCACCTCTGGCAGAAGAACAGACAGA
GTTCCCCACCTCCAAAACCCCTCTGTGGGCTTGCTCCAAAGTCTCTCATGGCCTGG
CTGCTCGCCAGCGACCGACCTCCACCCAAAGTTGTGCCTTCCAAGCAGATCCTTAC
TCAGATCATATGGAATGGAGCCAGAAACCATGGAGACCAAGTCAGTCACTGACTATT

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Figure 18b

TTAGCAAACCTGCACATGGGGTCAGTGGCATATTCCTGTACCAGCAAAAGGAAAAGCAA
GCTTGCTGAGGGAGAGGGGAAATGCCCCCTGAGTGGGAATGTACCAGGGAAAAACA
GCAAGGAACCAAAATAGCAGAGACGGAGGAGACACCAAAGGCAAAGTTGGCACTGT
ATCTTCAAGAGACAATCCACACCTCAGCACTTTTAACCTGGAGAGAACTGCCTTTCGC
AAGGACAGCCAAAGATGGTATGTGGCCTCTGATGGTGGGGTGGTAGAGAAAAGTGA
GTGGAAGCACCAGCCATGAAAGCCTTTCCCAGAGGTCCTGGTCTGGGGAACAGAGAG
GCTGAAGGGAAAGAGGATGGCACTATGGAAGGAGAGGCTGATGATGCTTCAGGACTT
GGTCAAGGGGAACGCTTCTGTGATATGGCCTGTGTAGCCTCAGCCAAAGACTTAG
ACAACCCTGAAGACACTGACTCTCCCACTTGTGACCATGCCACTAAGCTTCCTGAGGCT
GAAGACAATGTGGCCCGCCTTTGTGACTACCATTTGGCCAAGCGAATGTCATCCCTGC
AGAGTGAGGGCCATTTTTCTCTACAGAGCTCTCAAGGCTCTTCAGTGGACACAGGCTG
TGGCCCAGGCAGCAGTAGCAGTGCCTGTGCCACTCCTGTGGAATCGCCCCCTTGCCCA
TCCATGGGAAAGCACCTGATTCCAGATGCTTCTGGGAAAGGTGGGAGTTACATTTAC
CAGAGGAGAGAGTCGCTGGTCATCCCAACCATGGAGCCACCTTCAAGGAACTGCACCC
ACAGACAGAAGGGATGTGTCCACGCATGACAGTGCCTGCTCTGCACACAGCCATTAAT
GCCGACCCCTGTTTGGCACTTTGAGAGATGGATGCCATCGACTGCCCAAGATTAAGG
AAACCACAGTGTAG

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Figure 19

MMTNRDGRDYFINHMTQAIPFDDPRFDSCQIIPAPRKVEMRRDPVLGFGFVAGSEKPVVV
RSVTPGGPSEGKLIPGDQIVMINDEPVSAAPRERVIDLVRSCKESILFTVIQPYPSPKSAFISA
AKKARLKSNPVKVRFSSEEVIINGQVSETVKDNSLLFMPNVLKVYLENGQTKSFRFDCSTSI
KDVILTLQEKLSIKGIEHFSMLLEQRTEGAGTKLLLLHEQETLTQVTQRPSSHKMRCLEFRISF
VPKDPIDLLRRDPVAFEYLYVQSCNDVVQERFGPELKYDIALRLAALQMYIATVTTKQTQ
KISLKYIEKEWGLETFLPSAVLQSMKEKNIKKALSHLVKANQNLVPPGKKLSALQAKVHY
LKFLSDLRLYGGRVFKATLVQAEKRSEVTLLVGPRYGISHVINTKTNLVALLADFSHVNRI
EMFTEESLVRVELHVLDVKPITLLMESSDAMNLA CLTAGYYRLLVDSRRSIFNMANKKN
AGTQDTGTENKGKHNLLGPDWNCMPQMTTFIGEGEQEAQITYIDSKQKAVEMTDSTLCPK
EHRHLYIDNTYSSDELSQPLTQPGDAPCEADYRSLAQRSLLTSGPDTLKKAEQSPRGAKV
SFIFGDLALDDGMSPPTLGYERMLDENPEMLEKQRNLYISSANDMKNLDLTPDTSIQFVA
NSVYANIGDVKNFEAPEGIEEPLLHDICYAENTDDAEDEDEVSCCEEDLVVSEINQPAILDLS
GSSDDIIDLTTLPPPEGDDNEDDFLLRSLNMAIAAPPPGFRDSSDEEDTQSQATSFHENKEQ
GSSLQNEEIPVSLIDAVPTSAEGKCEKGLDPTVVSTLEALEALSEEQQKSENSGVAILRAYSP
ESSSDSGNETNSSEMTEGSELAAAQKQSESLSRMFLATHEGYHPLAEEQTEFPTSKTPSVGL
PPKSSHGLAARPATDLPPKVVPKQILHSDHMEMEPETMETKSVTDYFSKLHMGSVAYSC
TSKRKSKLAEGEGKCPLSGNVPGKKQQGTKIAETEEDTKGKVGTVSSRDNPHLSTFNLERT
AFRKDSQRWYVASDGGVVEKSGVEAPAMKAFPRGPGLGNREAEGKEDGTMEGEADDAS
GLGQGERFLSDMACVASAKDLNPEDTDSPTCDHATKLPEAEDNVARLCDYHLAKRMSS
LQSEGHFSLQSSQGSSVDTGCGPGSSSSACATPVESPLCPSMGKHLIPDASGKGGSYISPEER
VAGHPNHGATFKELHPQTEGMCPRMTVPALHTAINADPLFGTLRDGCHRLPKIKETTV*

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Figure 20

GGCACGAGTGAGCATGCCTGCCCTTTGCAAGCAGGTTTGGGTCTCACGCAGAGGAAAC
CAAAAGCAATAAGAGGGAGGGAAGGCAGAGCAACCAATCAAGGGCAGGGTGAGACT
CAAAACGAGCGGGCTCCCTGGGGAGCCAGACAGAGGCTGGGGGTGATGGCGGAGCTA
CAGCAGCTGCAGGAGTTTGAGATCCCCACTGGCCGGGAGGCTCTGAGGGGCAACCAC
AGTGCCCTGCTGCGGGTCGCTGACTACTGCGAGGACAACATATGTGCAGGCCACAGACA
AGCGGAAGGCGCTGGAGGAGACCATGGCCCTTCACTACCCAGGCACTGGCCAGCGTGG
CCTACCAGGTGGGCAACCTGGCCGGGCACACTCTGCGCATGTTGGACCTGCAGGGGGC
CGCCCTGCGGCAGGTGGAAGCCCGTGTAAGCACGCTGGGCCAGATGGTGAACATGCAT
ATGGAGAAGGTGGCCCGAAGGGAGATCGGCACCTTAGCCACTGTCCAACGGCTGCCCC
CCGGCCAGAAGGTCATCGCCCCAGAGAACCTACCCCTCTCACGCCCTACTGCAGGAG
AACCCTCAACTTTGGCTGCCTGGACGACATTTGGCCATGGGATCAAGGACCTCAGCAG
CAGCTGTCAAGAACAGGCACCCTGTCTCGAAAGAGCATCAAGGCCCTGCCACACCCG
CCTCCGCCACCTTGGGGAGACCACCCGGATTCCCGAGCCAGTGCACCTGCCGGTGGT
GCCCCAGGCAGACTCTCCGCCGCCTCCTCTGCGTCTTCCCTGGCCTCGGCCGGCAGCG
CCGAAGGTGTGCGTGGGGCCCCCACGCCCAAGGGGCAGGCAGCACCTCCAGCCCCAC
CTCTCCCCAGCTCCTTGGACCCACCTCCTCCACCAGCAGCCGTCGAGGTGTTCCAGCGG
CCTCCCACGCTGGAGGAGTTGTCCCCACCCCCACCGGACGAAGAGCTGCCCCTGCCAC
TGGACCTGCCTCCTCCTCCACCCCTGGATGGAGATGAATTGGGGCTGCCTCCACCCCCA
CCAGGATTTGGGCCTGATGAGCCCAGCTGGGTGCCTGCCTCATACTTGGAGAAAGTGG
TGACACTGTACCCATACACCAGCCAGAAGGACAATGAGCTCTCCTTCTCTGAGGGCAC
TGTCATCTGTGTCACTCGCCGCTACTCCGATGGCTGGTGGCAGGGCGTCAGCTCAGAG
GGGACTGGATTCTTCCCTGGGAACTATGTGGAGCCCAGCTGCTGACAGCCCAGGGCTC
TCTGGGCAGCTGATGTCTGCACTGAGTGGGTTTCATGAGCCCCAAGCCAAAACAGCT
CCAGTCACAGCTGGACTGGGTCTGCCACCTCTTGGGCTGTGAGCTGTGTTCTGTCCTT
CCTCCCATCGGAGGGGAGAAGGGGTCTGGGGAGAGAGAATTTATCCAGAGGCCTGCT
GCAGATGGGGAAGAGCTGGAAACCAAGAAGTTTGTCAACAGAGGACCCCTACTCCAT
GCAGGACAGGGTCTCCTGCTGCAAGTCCCAACTTTGAATAAAACAGATGATGTCCTGT
GAAAAAAAAAAAAAAAAAAAAA

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Figure 21

MAELQQLQFEIPTGREALRGNHSALLRVADYCEDNYVQATDKRKALEETMAFTTQALA
SVAYQVGNLAGHTLRMLDLQGAALRQVEARVSTLGQMVNMHMEKVARREIGTLATVQR
LPPGQKVIAPENLPPLTPYCRRTLNFGCLDDIGHGIKDLSTQLSRTGTLSRKSIKAPATPASA
TLGRPPRIPEPVHLPVVPDGRLSAASSASSLASAGSAEGVGGAPTPKGQAAPPAPPLPSSLD
PPPPA AVEVFQRPPTLEELSPPPPDEELPLPLDLPPPPPLDGDELGLPPPPPGFGPDEPSWVP
ASYLEKVVTLYPYTSQKDNELSFSEGTVICVTRRYSDGWCEGVSSEGTGFFPGNYVEPSC*

Figure 22a

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TTTCCAGCCAACGCCAAACAGTGACTGTTGACAATTTTCATATTGTCATCAGGGGAACC
AAGGCTTATTTCAGATGCCTATTTTCAGAACCTAGGACAGTTCCATTGAAAAGGCGCAGG
CGTTCCGGGCTGGCTGACTAGATGGATCAGGCCTGGCTGCCTGATGGCTATATTCTCCT
TCCTCCCTCTCCACTTCCATCTCAACCCTTGAGGCTGCATATTGAATAGTTGGAGAATT
CAGTGAACCTAAGAGATGCAAATGCACAGTACAAAATTCAAATGTCCAATTCGGGGCA
GGGCTGCATCTAACTTTAATGGCAACCACTGCATGTGATGTCTGGGGACTCTATAGAT
ACATGGCCTCAGACCCTGAAGACATCTGGATTCTGTCACTGGATTGTTACAAAAGTGA
GGCTGAACTTTCCACAGGACGAAGTCTTCAGGCTGGCCGCCTCCCTCGGGAACCTGGG
GCTTGAGCCAGGTGCCGCCCTATGGATGGGAGATGACGGCAAACCGAGATGGGCGAG
ACTACTTCATCAATCACATGACACAGGCAATCCCTTTTGACGACCCTCGGTTAGAGAG
CTGCCAAATCATCCCTCCGGCTCCTCGGAAGGTGGAGATGAGAAGGGACCCCGTGCTG
GGATTTGGTTTTGTGGCAGGCAGTGAAAAGCCAGTGGTCGTTGCTCAGTAACACCAG
GTGGCCCTCTGAAGGCAAGCTGATCCCGGGAGATCAGATTGTAATGATTAATGATGA
ACCGGTCAGCGCTGCACCCAGAGAGCGGGTCATCGATCTGGTCAGAAAGCTGCAAAGA
ATCGATACTCCTCACTGTCAATTCAGCCTTACCCTTCTCCCAAATCAGCATTATTAGTGC
TGCAAAAAAGGCAAGATTAAAGTCCAATCCTGTCAAAGTACGCTTCTCTGAGGAGGTC
ATCATCAACGGCCAAGTGTGCGAAACTGTTAAGGACAACCTCACTTCTTTTTATGCCAA
ATGTTTTGAAAGTCTATCTGGAAAATGGGCAGACCAAATCATTTGTTTTGACTGCAGC
ACTTCCATTAAAGGATGTCATCTTAACCTTCAAGAGAAGCTCTCCATCAAAGGCATTGA
ACACTTCTCTCATGCTGGAGCAGAGGACAGAAGGGGCTGGAACGAAGCTGCTCTTG
CTTCATGAACAGGAGACTCTAACTCAGGTGACACAGAGGCCCAGCTCCCATAAGATGA
GATGTCTTTTCCGAATTAGCTTCGTCCCAAAAGATCCAATTGACCTTTTAAGGAGAGAT
CCAGTTGCTTTTCGAGTATCTCTATGTTTCAGAGTTGTAACGATGTGGTTCAGGAGCGATT
TGGGCCGGAGCTGAAATATGACATAGCCCTGCGGCTGGCCGCATTACAAATGTACATT
GCAACCGTTACCACCAAGCAAACGCAGAAAATCTCCCTCAAATACATCGAAAAAGAA
TGGGGATTAGAGACTTTTCTTCCCTCTGCTGTGCTGCAAAGCATGAAAGAGAAGAACA
TAAAGAAAGCACTTTCACACCTTGTCAAAGCAAATCAAAACTTGGTACCACCGGGTAA
AAAGCTCTCTGCACTACAAGCCAAGGTCCATTATCTCAAGTTCCTCAGTGACCTACGAT
TGTATGGGGGCCGTGTGTTCAAGGCAACATTAGTGCAGGCAGAAAAGCGCTCGGAAGT
GACTCTCCTGGTTGGGCCCCGGTATGGCATAAGCCATGTCATCAACACCAAAAACCAAT
CTGGTGGCTCTTTAGCCGACTTTAGCCACGTCAACAGGATCGAAATGTTTTCCGAGGA
GGAGAGCTTGGTGCGGGTAGAACTCCACGTGCTAGATGTGAAGCCTATCACGCTTCTG
ATGGAATCCTCAGATGCCATGAACCTGGCCTGCTTGACGGCTGGATACTACCGGCTGC
TTGTTGATTCCAGGAGGTCGATATTTAACATGGCCAACAAGAAAAACACAGCGACCCA
GGAAACAGGACCTGAAAACAAGGGGAAGCATAACCTCCTTGGCCCAGATTGGAAGTGA
TATACCCCAAATGACCACCTTTATTGGCGAAGGGGAACAAGAAGCCCAGATAACATAC
ATAGATTCAAAGCAGAAGACGGTGGAGATCACAGACAGCACCATTGTGTCCAAAAGAG
CACCGGCACTTGTACATAGACAATGCCTATAGTTCAGATGGACTTAACCAGCAGCTGA
GCCAGCCCGGGGAGGCCCCCTGTGAGGCAGACTACAGAAGTCTAGCTCAGCGGTCCTT
ATTGACCCTCTCAGGACCAGAACTCTGAAGAAAGCACAGGAATCTCCGAGAGGAGC
TAAAGTGTCTTTATTTTTGGAGACTTCGCCTTGGATGATGGTATTAGTCCCCCAACCC
TTGGCTATGAAACGCTACTAGATGAGGTCTGAAATGCTGGAGAAGCAGAGAAATCT
CTACATTGGCAGTGCCAATGACATGAAGGGCCTGGATCTCACTCCAGAGGCAGAGGGC

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Figure 22b

ATCCAGTTTGTGGAAAATTCTGTTTATGCAAACATAGGCGATGTGAAGAGCTTCCAGG
CCGCGGAGGGGATCGAGGAACCCCTCTTGCATGACATCTGTTATGCAGAAAACACTGA
TGACGCGGAGGACGAGGACGAGGTGAGCTGCGAGGAGGACCTCGTGGTGGGGGAGAT
GAACCAGCCGGCCATCCTCAACCTGTCTGGGTCAAGCGATGACATCATTGACCTCACA
TCCCTGCCCCCTCCAGAAGGTGATGACAATGAGGATGACTTCCTGTTGCGTTCTTGAA
CATGGCCATTGCCGCACCCCCACCTGGCTTTAGAGACAGTTCAGATGAAGAGGACTCT
CAGAGCCAGGCAGCTTCTTCCCCGAGGACAAGGAGAAAGGCAGCAGCCTGCAAAAT
GATGAGATCCCCGTGTCCCTCATTGACGCTGTGCCACCAGCGCCGAAGGCAAGTGTG
AGAAGGGACTGGATAATGCCGTCGTCTCCACGCTGGGAGCTCTAGAGGCTCTATCCGT
GTCAGAAGAACAGCAGACCAGTGACAATTCAGGTGTAGCCATCTTGCGGGCTTATAGT
CCTGAGTCTTCGTCAGACTCGGGCAATGAAACTAACTCTTCTGAAATGACTGAGAGTT
CTGAACTGGCCACAGCACAAAAACAGTCAGAAAACCTCTCCCGCATGTTCTTGGCCAC
TCACGAAGGCTACCACCCCTTGCAGAAGAGCAGACCGAGTTCCCGGCCTCCAAGACC
CCCGCTGGGGGCTTGCCCTCAAAGTCCTCGCACGCCCTGGCTGCTAGGCCAGCAACCG
ACCTCCCGCCCAAAGTTGTGCCTTCCAAGCAGTTACTTCACTCAGACCACATGGAGAT
GGAGCCTGAAACTATGGAGACTAAGTCGGTCACTGACTATTTAGCAAACCTGCACATG
GGTTCGGTGGCATACTCCTGCACTAGCAAAAAGGAAAAAGCAAGCTGGCCGATGGTGAG
GGGAAGGCACCCCTAATGGGAACACAACAGGAAAAAACAGCAGGGGACCAAAAC
GGCAGAGATGGAGGAGGAGGCCAGTGGTAAATTTGGTACTGTGTCTTACGAGACAGT
CAACACCTGAGCACTTTTAATCTGGAGAGAACTGCCTTTCGCAAGGACAGTCAAAGAT
GGTATGTGGCCACTGAAGGTGGGATGGCTGAAAAAAAGTGGATTAGAAGCAGCAACA
GGGAAAACCTTTCCAAGAGCTTCTGGTCTTGGGGCAAGGGAGGCCGAAGGGAAGGAA
GAAGGAGCTCCTGATGGAGAAACAGTGATGGCTCAGGACTTGGTCAAGGGGACCGC
TTCTTAAGTACGCTGACCTGTGCATCTTCAGCCAAAGACTTAGATAACCCAGAGGACG
CTGACTCGTCCACCTGCGACCATCCTTCCAAGCTTCTGAGGCTGATGAGAGTGTGGCC
CGCCTTTGTGACTACCACTTGGCCAAGCGGATGTCATCACTGCAAAGCGAGGGCCATT
TTTCTCTGCAGAGCTCCCAAGGCTCTTCAGTGGATGCAGGCTGTGGCACAGGCAGCAG
TGGCAGTGCCTGTGCCACACCCGTGGAGTCGCCGCTCTGCCCCTCCCTGGGGAAGCAC
TTGATTCTGACGCTTCTGGGAAAGGCGTGAATTACATTCCTTCAGAGGAGAGAGCCC
CTGGGCTTCCCAACCACGGAGCCACCTTTAAGGAACTGCACCCACAGACAGAAGGGAT
GTGTCCACGGATGACAGTGCCTGCTCTGCACACAGCCATTAACACCGAACCCCTGTTT
GGCACATTGAGAGATGGATGCCATCGGCTCCCCAAGATTAAGGAAACACAGTGTAGC
TTTGACAGAGCCTGGGAAGGAGAGACGAGGAGGCATGCCTTCAGCTTGGTCTCAACAT
CCTGAAGCTGATCCCATCCTGCTACCATCAAACATTCAGTTCGGAATCAAAGGTGCCAA
TTCCAAATCAAGACCCTAATGATTTCTCCCAAGCAAATCAGGCATACGGAGAGGCTGT
GAGCTGGCGGCCACCGGATCTGAGAGGGGGGAGCCTCAGGACACCTCCCAGCCAGAA
GGCTCTGAGACATAGCAGCAGTATCCTCTCCGGATCTGTGATTTGGAGACCTTCCGA
GAGAGAACCAAGGGTGCAGTCAGCTTAAAGTGTCCAGGCATCACAGAAGCACAGGAG
GCCAGTTCTGAAAGGCGAGCAGAACTCCCCCTGGGGAGGAAGCTCACAAAAGTTTTT
CCCAAAGCTCAATGCACCTGAGCTCTGAGGGGAGGTTTCACAAAAGGTCCCCAGTGGC
TCATAAAGACTCAAAGCTGTATAGGACATTACCCTTGCGGAAGCTGGAGGGCAGCAAT
TGGAGATGCCGGGACCCTTCAGCTATTGCTTCCTGAACCGAGGGCAGGATGAAGATG
GTGAGGAAGAAGAGGAGAGGGGAGAGGCCACCGTCCAGGTCTCTTGCTCTATAGAC
CACAGGTGACTCAAGCCATGCCAGAACCAAGCAGCCCATGCCTGGCTGTGGCGATTCA
GAAGCAACGAGGGGAGCTATCCAGAGGGTCAGTGCTGAAGGTCTGGGCAGAAGACCT
GCGAGACCCAGATGACTTGGACTTCAGCAACCTGGCTTTTGATGCCCGGATTGCAAGA

Figure 22c

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ATAAATGCCCTAAAGGAGAGCACATATGCAATGCCTGATGGGTTCCCTGCAGCCCCAAA
ATGATGCCAATGAGCTGCTCTGTCTCGTCAGGGCAACCAAGGAGAAGAGGGAGGAGT
CACGCCCTGAAGCGTACGACCTTACACTTTCTCAGTACAAGCAACTGTTATCCATTGAG
TCCAGACAGTTGGGAAGTGCCTGTAGGAAAAATGGCGATGGCTGAGAAAAGCCCGGAG
GAGATGCTCCTAGCTATGACTTCCAGCTTTCAAGTGCTCTGTTGCCTAACAGAAGCTTG
CATGCGATTAGTTAAAGTCGTGAACTCAGAAACACAGCGGCAGGAAATTGTAGGGAA
GATCGATGAAGTGGTCATAAATTACATTTTGTCTACTGAAAGCTGCCGAAGCAGCCACT
GGAAAGAACCCTGGGGACCTAATGTTGGACTCTCGGCGCGACACTCAACCACCATGG
CCGCTCTCGTAAGCACACTGACACGTTCTCTCAAGAGGCTTTTAAACAAATAAATATG
GAAGTCACGTCATAATCTACCTTTGCAAAGCCATACATGAACTTTTATTTACTTTGTGT
GTATGATGAACAGATGTCTCCTTTCTCTCTGTATATTTTGTATTTTATATAAAAATA
GGAGATAAAAAGTCACACTGATGAAATGTTGAAATGTACTAATCAGATGTATTCTGTTT
ATATTATACATATATATACACGTAAAAGAAATATCCAAGAAAGTGATGACATTTGGCT
ATTTTTCATATAGTTAAAACTCCAGGTATATGATGTGAAATTTTAAATTCTACCATGTT
AGAGCAAAACAATGAATCCTATCCCCCTTTCTTCCAAGTAGCTACTTGAAACCATATC
ATTCATATTTAGAAGTAAAACACAAAAACAAAAAAGAGAGAGAAAAAGAAAAAGAAATCA
CAATGTATATAAAACAGTACTTATGTTTTTAAATTATGATTTTTTAAGCATTGGAAATAG
CAAAAAGACATTTAAATTCAAGAAGCTATTATGAATTACTAGAGAATATATCTGTAA
TAAATTAATTTTTTGCTCATAGTATTTGGTTACTGGATGCTTTCTTCCAAGAATCCACA
TATTTAATTTGGGTTTTTGCTACTGGGGCTACAAATTGGTGGGGATGGATTCTACTGTG
TCAGCACAAATGCTCTTCACAGTGGTTCTAGCATTAAAAAACTTCCCGGGGAGAAGA
ACAGAGGGGATGATGGGCAGTTTCCTAGGTAACACCTAGAGTTATAGAATATCTCATT
ACATAAAATGTATGGAATTAATAATACCAAAATTAATTATTTGATGGAAAGATCTGCT
TTGACTAAATGTCAAAAATCTGCAAACCAAGACATTATCTTCCCCTCATCCCAACTCA
ACTACGAAACTTAAATTCCTTTAGAGTGATAGGACATTTAGTAAAGTATTTGCAA
CTTAAAAAAGGAACATTTAATGATCATCAAAATTAAGTACAGATTGAGTAATGTAGA
CCAGACCACACACCAGCACCTGTGAGTCTCATCTCAGATCACAGCTCTCAGCATAGGG
CTTCATGCATCACCGCCTCTACAGAGGCTAAGGCTGCCAGTCAAATTTGGAATTATAG
CGTAGTACTGGGACAAAATCTCAAATCTTGGATGTTCCAGAAAATCAGGGAGTGATGG
CTACTGTAATCATGGGAGCCATGAGTAAATAGTTAAGTATTTATTAATAAATACTTA
ATCTGGATTGGCTGATAAAAATATGAAATCT

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Figure 23

MTANRDGRDYFINHMTQAIPFDDPRLESCQIIPAPRKVEMRRDPVLGFGFVAGSEKPVVV
RSVTPGGPSEGKLIPGDQIVMINDEPVSAAPRERVIDLVRCKESILLTVIQPYPSPKSAFISA
AKKARLKSNPVKVRFSEEVINGQVSETVKDNSLLFMPNVLKVYLENGQTKSFRFDCSTSI
KDVILTQEKLSIKGIEHFSMLLEQRTEGAGTKLLLLHEQETLTQVTQRPSSHKMRCLFRISF
VPKDPIDLLRRDPVAFEYLYVQSCNDVVQERFGPELKYDIALRLAALQMYIATVTTKQTQ
KISLKYIEKEWGLETFLPSAVLQSMKEKNIKKALSHLVKANQNLVPPGKKLSALQAKVHY
LKFLSDLRLYGGRVFKATLVQAEKRSEVTLLVGPRYGISHVINTKTNLVALLADFSHVNR
EMFSEEEESLVRVELHVLDVKPITLLMESSDAMNLA CLTAGYYRLLVDSRRSIFNMANKKN
TATQETGPENKGKHNLLGPDWNCIPQMTTFIGEGEQEAQITYIDSKQKTVEITDSTMCPKE
HRHLYIDNAYSSDGLNQQLSQPGEAPCEADYRSLAQRSLLTLSGPETLKKAQESPRGAKVS
FIFGDFALDDGISPPTLGYETLLDEGPMELEKQRNLYIGSANDMKGLDLTPEAEGIQFVENS
VYANIGDVKSFQAAEGIEEPLLHDICYAENTDDAEDEDEVSCCEDLVVGEMNQPAILNLSG
SSDDIIDLTSLPPPEGDDNEDDFLLRSLNMAIAAPPPGFRDSSDEEDSQSQAASFPEDKEKGS
SLQNDIEPVSLIDAVPTSAEGKCEKGLDNAVVSTLGALEALSVSEEQQTSDNSGVAILRAYS
PESSSDSGNETNSSEMTESELATAQKQSENLSRMFLATHEGYHPLAEEQTEFPASKTPAGG
LPPKSSHALAARPATDLPPKVVP SKQLLHSDHMEMEPETMETKSVTDYFSKLHMGSVAYS
CTSKRKSKLADGEGKAPPNGNTTGKKQQGTKTAEMEEEASGKFGTVSSRDSQHLSTFNLE
RTAFRKDSQRWYVATEGGMAEKKWIRSSNRENLSKSFWSWGKGGRREGRRSS

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Figure 24

AGCGGGGCTCCATTGTGCTCGGCGGGGGCCGGGAAGCCAAAGGAGGTGGGCTCGGGC
CCCTGCGCTGCTCCCCGGCGGCTGCGCCCCAGCTAGCTGCCAGCCTGGAAATGGCTC
CGCTGCTGCTCCTCGGGAACGAATCGATCCTTCCCAGCCTTCTCTGCCTGCTCTCCA
CCTCCTCTCTGCTCCGAGTCTTAGGAGGACGAACATTCAAAGGACAGATTCCAATGTG
GTGTGCCGTGCACATCGGGAGCGGCTGGGGTTTGCACTTCGAGATTTCTTCTATATAAT
TTTTTTTTTTAAACGTAAGGGAGGCAGTAGCATTGCTGCCTGTAGGATTTTTATCA
AGTGCACGTGCGGTTGGGTTGCACGNTCCACCCCCAGGGACCTGGTGTGGTGAAATTT
GAACCCACCGCCTTAGCCCCAAAAGGCCGAGTAACCTGGCTGCCTGAGTGTCTGGAA
GACGTGAGCGAAATGACCAGCGAACTCATTTTTATCAGACTTGCTGAAGCTGGCTTTT
GCGTTTTTCTACACGTACGCTTAATTTTGTGGAATAGTTAAGTGCTATATTCTCCGCGC
AACCTTTTCAAATTCCAAATGTTTGAACATTTTGGTGTGAGCGCGAGTGAAATCATTTT
ACCGACAAGAACTAACTGAATTGTCTGCCTTGTGAGTTGCCTCCGGAAAAGATCTCG
GGGGTGGAAAAGCAACTGCAAAATAACAGACGGAGAAAATTCCTTGGAAGTTATTTCT
GTAGCATAAGAGCAGAACTTCAGAGCAAGTTTTATTGGGCAAAATGGGGGAGCAA
CCTATCTTCAGCACTCGAGCTCATGTCTTCCAGATTGACCCGAACACAAAGAAGAACT
GGGTACCCACCAGCAAGCATGCAGTTACTGTATCTTATTTTTATGACAGCACAAAGAAA
TGTGTATAGGATAATCAGTTTAGATGGCTCAAAGGCAATAATAAATAGCACCATCACA
CCAAACATGACATTTACTAAAACATCTCAAAAGTTTGGCCAATGGGCTGATAGCCGGG
CAAACACTGTTTATGGACTGGGATTCTCCTCTGAGCATCATCTTCAAAAATTCGCAGAA
AAGTTTCAGGAATTTAAGGAAGCTGCTCGGCTTGCAAAGGAGAAGTCGCAGGAGAA
ATGGAGCTGACCAGTACCCCTTCACAGGAATCAGCAGGAGGAGATCTTCAGTCTCCTT
TGACACCAGAAAGTATCAATGGGACAGACGATGAGAGAACACCCGATGTGACACAGA
ACTCAGAGCCAAGGGCTGAGCCAACCTCAGAATGCATTGCCATTTCCACATAGGTACAC
ATTCAATTCAGCAATCATGATTAAGTAAGGTGGATAAATATGGAAGTTCATTTGGTTTC
AGAAACTCTTGAAGTTACAACCTTTGAGTGAAAAATCTCAGGTCAGACTCCTTTAATTT
ATTGTTCTTGGTTGCTCAAGTTGACTGAATTACTATATTTCCATTATCTATGTGGAAAA
AGGAGCATTGAGCTAATTATAGGAGAAAATTTTTAAATGGAGAAAAATATAATTCCTTT
CTATCTATATTTTAAAGATCCCTTTTGTTAACCCGTTTTCTGTNTTATATATGTTATGT
AAGATTTATAATGTGTAATTAGAAACATAGAATTTCTACTCTGAAGGAAAGCTTTACC
ACAGGCCTACAGAGTTTTACAGAAGACAGGGTACCAAGCACGAGCCTGTTAGCATTG
ATGGCAGATGCCAGCAGAAGGAAGGCTTGACTTCCTAATTCTGTATTCTAAAAGATAC
ATCATGTTCTAAATGCATTTCAAACATTAGTTATTGGCCGTACCGTGGCATTACTGGAC
TGTAACATGAATGTGAAATGGCACTATTGAAAATATTTTTTAAAGCCCATCTACCTT
AACACTAATTTTACCCTTATTTAAATGCTTTTTACTAAATAGTTTTAGGTAAATTA
GAAAATAGGGGTTTTTGAAGTGCACATTTTTTGAAGAACCAAGTTTAGAAAATTATA
TTCTTTGACAGATTAAAAATTGCAAAGTGAGATATTTCAAACCTCTCCTAGGTGAGTTT
TATTGTGTTTGAACCTGCATTAATAGGGGCATAGGAT

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Figure 25

MGEQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAINSTITP
NMTFTKTSQKFGQWADSRANTVYGLGFSSEHLSKFAEKQEFKEAARLAKEKSQEKME
LTSTPSQESAGGDLQSPLTPESINGTDDERTPDVTQNSEPRÆPTQNALPFPHRYTFNSAIMI
K*

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Figure 26

GAATTCGGCACGAGTCTGCCTTGTTGAGTTGCCTCCGGAAAAGATCTCGGGGGTGGAA
AAGCAACTGCAAAATAACAGACGGAGAAAATTCCTTGGAAGTTATTTCTGTAGCATAA
GAGCAGAACTTCAGAGCAAGTTTTTCATTGGGCAAAATGGGGGAGCAACCTATCTTCA
GCACTCGAGCTCATGTCTTCCAGATTGACCCGAACACAAAGAAGAACTGGGTACCCAC
CAGCAAGCATGCAGTTACTGTATCTTATTTTTATGACAGCACAAGAAATGTGTATAGG
ATAATCAGTTTAGATGGCTCAAAGGCAATAATAAATAGCACCATCACACCAAACATGA
CATTTACTAAAACATCTCAAAAGTTTGGCCAATGGGCTGATAGCCGGGCAAACTGT
TTATGGACTGGGATTCTCCTCTGAGCATCATCTTTCAAAATTCGCAGAAAAGTTTCAGG
AATTTAAGGAAGCTGCTCGGCTTGCAAAGGAGAAGTTCGAGGAGAAGATGGAGCTGA
CCAGTACCCCTTCACAGGAATCAGCAGGAGGAGATCTTCAGTCTCCTTTGACACCAGA
AAGTATCAATGGGACAGACGATGAGAGAACACCCGATGTGACACAGAACTCAGAGCC
AAGGGCTGAGCCAACTCAGAATGCATTGCCATTTCCACATAGTTCAGCAATCAGCAAA
CACTGGGAGGCTGAGCTAGCTACCCTCAAAGGCAACAATGCCAACTCACTGCAGCCC
TGCTGGAGTCCACTGCCAATGTGAAGCAGTGGAAGCAACAGCTTGCTGCGTACCAGGA
GGAAGCAGAGCGGCTGCACAAGCGGGTCACTGAGCTGGAGTGTGTTAGTAGTCAAGC
AAACGCTGTGCACAGCCACAAGACAGAGCTGAACCAGACAGTGCAGGAACTGGAAGA
GACCCTGAAAGTAAAGGAAGAGGAAATAGAAAGATTAAAAACAAGAAATCGATAATGC
CAGAGAACTCCAAGAACAGAGGGACTCTTTGACTCAGAACTACAGGAAGTTGAAAT
TCGAAATAAAGACCTGGAGGGGCAGCTGTCTGACCTAGAACAGCGCCTGGAGAAGAG
CCAGAACGAACAAGAGGCTTTCCGCAGTAACCTGAAGACACTCCTAGAAATTCTGGAT
GGAAAAATATTTGAACTAACAGAATTACGAGATAATTTGGCCAAGCTACTGGAATGCA
GCTAAAGAGAGTGAAATTTTCAGTGCCAATAGATGGAGAGATGCTGTCTGTCTTCCTAG
GACTGTTTGGGCTCCGTACCAAGATTGCACAAAATTTTTTGAATATCATTCCTCCAGGA
GGAGGGTGTTTTGAAGATTGGAATTGTATATTTTCAGTATAAATTTTTGAATTTAGCTTA
TAGCTAATTGGGAAAAAAAAAAAAAAAAAAAAA

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104659-990000

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Figure 27

MGEQPIFSTRAHVFQIDPNTKKNWVPTS KHAVTVSYFYDSTRNVYRIISLDGSKAIINSTITP
NMTFTKTSQKFGQWADSRANTVYGLGFSSEHLSKFAEKQEFKEAARLAKEKSQEKME
LTSTPSQESAGGDLQSPLTPESINGTDDERTPDVTQNSEPRAEPTQNALPFPHSSAISKHWEA
ELATLKGNNAKLTAALLESTANVKQWKQQLAAYQEEAERLHKRVTELEC VSSQANAVHS
HKTELNQT VQEEETLKVKEEEIERLKQEIDNARELQEQRDSL TQKLQEVEIRNKDLEGQL
SDLEQRLEKSQNEQEAFRSNLKTLLEILDGKIFELTEL RDNLAKLLECS*

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Figure 28

GGCTTGGCCACGCGTCGACTAGTACGGGGGGGGGGCGTCGGAGCGGCCGCACGAGCAG
CGCCGGAGATGGGAGAACAGCCCATCTTCACCACGCGAGCGCACGTCTTCCAGATTGACC
CCAGCACCAAGAAGAAGTGGGTGCCGGCAAGCAAGCAGGCCGTCACGGTTTCTACTTCT
ATGATGTCACCAGGAACAGCTATCGGATCATCAGTGTGGATGGAGCCAAGGTGATCATAA
ACAGCACTATCACCCCGAACATGACTTTACCAAAAACGTACAGAAAGTTTCGGGCAGTGGG
CTGACAGCAGAGCCAACACCGTGTTCCGGTTTGGGATTCTCCTCCGAGCTGCAGCTCACGA
AGTTTGCAGAGAAAGTTCCAGGAGGTAAGAGAAGCTGCCAGGCTAGCCAGAGACAAGTCC
CAGGAGAAAAACCGAGACCTCCAGCAATCATTCCCAAGCATCCAGCGTCAATGGCACAGA
CGACGAAAAGGCCTCTCACGCGAGCCAGCCGACACTCACCTCAAGTCTGAGAATGACAA
GCTGAAGATCGCGCTGACACAGAGTGCTGCCAATGTGAAGAAGTGGGAGATGGAGCTGC
AGACCCTGCGGGAGAGCAACGCCCGGCTGACCACGGCACTGCAGGAGTCGGCGGCCAGC
GTGGAGCAGTGGAAGCGGCAGTTCTCCATCTGCAGGGACGAGAATGACAGGCTCCGCAG
CAAGATCGAGGAGCTGGAAGAACAGTGCAGCGAGATAAACAGGGAGAAGGAGAAGAAC
ACACAGCTGAAGAGGAGGATCGAGGAGCTGGAGTCAGAGGTCCGAGACAAGGAGATGG
AGTTGAAAGATCTCCGAAAACAGAGTGAAATCATACCTCAGCTCATGTCCGAGTGTGAAT
ATGTCTCTGAGAAGTTAGAGGCGGCCGAAAAGAGACAATCAAACTTGAAGACAAAGTG
CGGTCTCTAAAGACAGACATCGAGGAGAGTAAATACCGACAGCGCCACCTGAAGGGGGA
GCTGAAGAGCTTCCTTGAGGTGCTGGATGGAAGATCGACGACCTCCATGACTTCCGTAG
AGGACTCTCCAAGTTAGGCACAGATAACTAGGGCGGGCGGAGCAAGTGTGTGTGAGAG
GTGTGGTAGACGTAGGACATTCTCCATTTGCTTCTGTAAATGCAGGTGCGATCTGTCTGTC
TCCAGACCAATTGTGCCGTCCGCTCACTCCTCCAGAATAGGAAATCTCTCGCTTCTCTGGC
TTTGTGAGGTCATGGACAGCTGGAAGCTTCTGACTCAGGAATCCAGAACTTGGTCTACCTT
AGCCGTTTACGCAGTCAGGGCAGGGATGTTTAGATCTTCCCTTAAGGGCTGTTGTAACCTT
ATGAACCGGGGATGGGGGAGTATTTTCTAATCCAAGTACCATTATCCTTTACAGCAGGCC
CTCGGGTGCTTCTGCTGCGTGGCATTCAAGTGTATGTGACTCTCCAGCAGGTTCTAGACCA
CGGGCATGTGGAGGGAGCATCTTTTCCCAGTATGCATTTTGTGCTTTAGCAGATGTGACA
TGACATTGTCAACCACAAAGTTCACACTCAAAAACGTGCACAACTGACTTACTCAAAAAGA
AATAATTGTAAAAA

Figure 29

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MGEQPIFTTRAHV FQIDPSTKKNWVPASKQAVTVSYFYDVTRNSYRIISVDGAKVIINSTITP
NMTFTKTSQKFGQWADSRANTVFGLGFSSSELQLTKFAEKFQEVREAARLARDKSQEKTTET
SSNHSQASSVNGTDDEKASHASPADTHLKSENDKLKIALTQSAANVKKWEMELQTLRESN
ARLTTALQESAASVEQWKRQFSICRDENDRLRSKIEELEEQCSEINREKEKNTQLKRRIEEL
ESEVRDKEMELKDLRKQSEIIPQLMSECEYVSEKLEAAERDNQNLEDKVRSLKTDIEESKY
RQRHLKGELKSFLEVLDGKIDDLHDFRRGLSKLGTDN*GG

GGCTTGGCCACGCGTCGACTAGTACGGGGGGGGGGCGTCGGAGCGGCCGCACGAGC
AGCGCCGGAGATGGGAGAACAGCCCATCTTCACCACGCGAGCGCACGTCTTCCAGATT
GACCCACGACCAAGAAGAACTGGGTGCCGGCAAGCAAGCAGGCCGTCACGGTTTCC
TACTTCTATGATGTCACCAGGAACAGCTATCGGATCATCAGTGTGGATGGAGCCAAGG
TGATCATAAACAGCACTATCACCCGAACATGACTTTCACCAAAACGTCACAGAAGTT
CGGGCAGTGGGCTGACAGCAGAGCCAACACCGTGTTCGGTTTGGGATTCTCCTCCGAG
CTGCAGCTCACGAAGTTTGCAGAGAAGTTCCAGGAGGTAAGAGAAGCTGCCAGGCTA
GCCAGAGACAAGTCCCAGGAGAAAACCGAGACCTCCAGCAATCATTCCCAAGAATCT
GGGTGTGAAACCCCGTCTTCCACTCAGGCATCCAGCGTCAATGGCACAGACGACGAAA
AGGCCTCTCACGCGAGCCCAGCCGACACTCACCTCAAGTCTGAGAATGACAAGCTGAA
GATCGCGCTGACACAGAGTGCTGCCAATGTGAAGAAGTGGGAGATGGAGCTGCAGAC
CCTGCGGGAGAGCAACGCCCGGCTGACCACGGCACTGCAGGAGTCGGCGGCCAGCGT
GGAGCAGTGGAAGCGGCAGTTCTCCATCTGCAGGGACGAGAATGACAGGCTCCGCAG
CAAGATCGAGGAGCTGGAAGAACAGTGCAGCGAGATAAACAGGGAGAAGGAGAAGA
ACACACAGCTGAAGAGGAGGATCGAGGAGCTGGAGTCAGAGGTCCGAGACAAGGAG
ATGGAGTTGAAAGATCTCCGAAAACAGAGTGAAATCATACCTCAGCTCATGTCCGAGT
GTGAATATGTCTCTGAGAAGTTAGAGGCGGCCGAAAGAGACAATCAAACTTGAAG
ACAAAGTGCGGTCTCTAAAGACAGACATCGAGGAGAGTAAATACCGACAGCGCCACC
TGAAGGGGGAGCTGAAGAGCTTCCTTGAGGTGCTGGATGGAAAGATCGACGACCTCC
ATGACTTCCGTAGAGGACTCTCCAAGTTAGGCACAGATAACTAGGGCGGGGCGGAGC
AAGTGTGTGTGAGAGGTGTGGTAGACGTAGGACATTCTCCATTTGCTTCTGTAAATGC
AGGTGCGATCTGTCTGTCTCCAGACCAATTGTGCCGTCCGCTCACTCCTCCAGAATAGG
AAATCTCTCGCTTCTCTGGCTTTGTGAGGTCATGGACAGCTGGAAGCTTCTGACTCAGG
AATCCAGAACTTGGTCTACCTTAGCCGTTTACGCAGTCAGGGCAGGGATGTTTAGATCT
TCCCTTAAGGGCTGTTGTAACCCTATGAACCGGGGATGGGGGAGTATTTTCTAATCCA
AGTACCATTATCCTTTACAGCAGGCCCTCGGGTGCCTTCTGCTGCGTGGCATTCAAGTGT
ATGTGACTCTCCAGCAGGTTCTAGACCACGGGCATGTGGAGGGAGCATCTTTTCCCAG
TATGCATTTTGTGCTTTAGCAGATGTGACATGACATTGTCAACCACAAAGTTCACACT
CAAAAACCTGCACAACTGACTTACTCAAAAAGAAATAATTGTAAAAAAAAAAAAAAAAAA
AA

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Figure 31

MGEQIFTTRAHVQIDPSTKKNWVPASKQAVTVSYFYDVTRNSYRIISVDGAKVIINSTITP
NMTFTKTSQKFGQWADSRANTVFGLGFSSELQLTKFAEKFQEVREAARLARDKSQECTET
SSNHSQESGCETPSSTQASSVNGTDDEKASHASPADTHLKSENDKLKIALTQSAANVKKW
EMELQTLRESNARLTTALQESAASVEQWKRQFSICRDENDRLRSKIEELEEQCSEINREKEK
NTQLKRRIEELESEVRDKEMELKDLRKQSEHPQLMSECEYVSEKLEAAERDNQNLEDKVR
SLKTDIEESKYRQRHLKGELKSFLEVLDGKIDDLHDFRRGLSKLGTDN*

TCCACAGCCAGGGAACAGCCAATCTTCAGCACCCGGGCGCACGTATTCCAGATCGACC
CCACTACAAAGCGGAAGTGGATCCCCGCCGGCAAGCACGCACTTACCGTGTCTATTT
CTATGATGCAACCCGAAATGTGTACCGCATCATCAGCATCGGGGGTGCCAAGGCCATC
ATCAACAGCACTGTCACTCCCAACATGACCTTCACCAAAACCTCTCAGAAAGTTCGGGC
AATGGGCAGACAGTCGAGCCAACACTGTCTACGGCCTAGGCTTTGCCTCTGAACAGCA
GCTGACCCAGTTTGTGAGAAGTTTCAGGAGGTGAAAGAAGCTGCCAGGCTGGCTCGA
GAGAAATCTCAAGATGGTGGAGAATTCAGTAGTACTGGCCTGGCCCTTGCCTCCCATC
AGGTTCTCCAAGCCCCTTGGTCAGACCAATGGTCCAGGCGAGGAAAAGCTGTTCCG
TAGCCAGAGTGGGACACCCCTGGCCCCACCGAGCGGGAACGGTTGAAGAAGATGCT
GTCAGAAGGCTCTGTAGGGGAAGTCCAGTGGGAAGCAGAGTCTTCGCGCTTCAGGAC
AGCAACCAGAGGTTGGCGGGAGCCCTTCGGGAAGCGAACGCAGCGGCCACTCAGTGG
AGGCAACAAGTGGAGGTCCAACGTGCAGAGGCTGAACTCTTGAGGCAGCGGGTAGCA
GAGCTGGAGGCCAGGTGGCTGTAGAGCCAGTCCGGGCAGGAGAGAAAGAAGCAACC
AGCCAGTCGGTGGAGCAGCTGGAGGCTCGGGTGCAGACCAAGGACCAGGAGATCCAG
ACTTTGAAGAATCAGAGCACTGGCACCCGAGAGGCTCCAGACACTGCCGAGCGCGAA
GAGACACAGCAGCAAGTTCAGGACCTGGAGACCCGGAATGCAGAGCTGGAGCAGCAG
CTGCGGGCGATGGAGTGCAACCTGGAGGAGGCGCGGGCCGAGCGGGAGCGCGCACGG
GCGGAGGTGGGCCGGGCTGCGCAGCTGCTGGATGTTTCGGCTGTTTGTAGCTCAGCGAGC
TGCGTGAAGGCTGGCACGCCTGGCAGAGGCAGCACCCCTAGTCTGCCATGGAGTGTCT
GCGGCCTCAAGGCGCCCTGGCAGGGGGCAGGGGACCCAGCTGTCTCTGAGCTTTGCA
CTGTGTAGAGTTTTCTAGAATCCTTGGGCAATGCTTCTACCCAGGTTACATTTCTACGT
GTGGCGTTGCTGTCCCTGGCTGCTGCTGCCCTGCGCCCCAGGGACACTGCGAGGGAAG
GCTGCACTAGTCATCCCCATGGGGCAACAGAGGCTTTGGGATCCTGAGACCTGAAGGC
CCTGTACTCATCCACCCCATTTCTCAAGTCAGACTGACAACTTCAAAGAGTGTACTG
AAGTCAGGGGCCACCAAGCACCAGGTTTACAGCTCAGTCCTGAGCCTCAGCCTGGGCTG
GCTCTTGGGGCCGAGATCTGGGAGGACGCGACCGTCGGACAGTGCTCCCTGCTTTCTG
CAGCTCAGCTTTGCGTTTGGCCTCCAGGTCTGGGTCTGCGGAAGGGAGCTGAGAATG
TAACTGGGCAGCTTCCAGGGACTGGCTCCCCCACCCTACCCGTCCCCAGGTCCCACC
CACCCTTACTGGCCACACTCTTATGCCTGTCCCTGCATACCCATGCCTCCCTATACTAC
CTTCCCCTCCAGGATCATCTGTTTCCGCTTGTTGATCTCTTTCTTTTCATCAAAATGCGA
AGCCTCCAGTTTCTAGGGGTGGGGAGGGGAACAGGTCAGTCAGGCCTGGGGCAGGAA
GCCCCGCCACCTCACCCTACCCCTACCCTGACAGGCTGGCCACACTTACTATTT
CGCACTCCCTTCGCACTACGTTGACCTGCGTGAGGATTTGTAGAACCTCAGCCTCCTCC
ACCACCAGCTCTGCCAGCTGCTGCTCTGCAGGGACAGGAAACACTGAGTTGGGCTGGG
AGTGCAACCAGCCCTCTGCACCCCAAGCTCTGGATGTCTGGATCCAACCAAATGTGGA
CTGATGATATTTAGAAAAAGCAAAATGCTGCCAAGCTTGGCAGCACATGCTTGTATC
ACAGCACTGGGAGGTGGAGGCAGGGGGATCACTCGTTTCAGCTGAGTTCCAGGCCAGC
TCTGTAGAGCAAGAATCTGTCTCAAATTAATGACTGAATAAACAAATGAACAAGTAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

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Figure 33

MSTAREQPIFSTRAHVFQIDPTTKRNWIPAGKHALTVSYFYDATRNVYRIISIGGAKAINST
VTPNMTFTKTSQKFGQWADSRANTVYGLGFASEQQLTQFAEKFQEVKEAARLAREKSQD
GGEFTSTGLALASHQVPPSPLVSTNGPGEEKLFRSQSADTPGPTERERLKKMLSEGSVGEV
QWEAEFFALQDSNQRLAGALREANAAATQWRQQLEVQRAEAELLRQRVAELEAQVAVE
PVRAGEKEATSQSVEQLEARVQTKDQEIQTLKNQSTGTREAPDTAEREETQQQVQDLETR
NAELEQQLRAMECNLEEARAERERARA EVGRAAQLLDVRLFELSELREGLARLAEAAP*

Figure 34b

TTGACAGTGCTCCCGTAGAAATCCTACATTATTTCAACCTCAGAGTTACAGTAAAGGC
AGGTTATAACCAGTCTTTCTTATTATTTAAGAATTTCCAGCCCTAGTGTTTTATGAAA
GTATTCCTGTGAATTTGACACCTTATGATCCTATATTCATCTAATTCCTTAATGAAATA
AAAATGTCCATGTGAGGTAGGTTATTTACAGCGATTGCAGGAGACATGGTGTTCTTCA
GAGTTCCCAAACCAGGATAGTTTCAAATAGGTTTTTCATGGCTTCTGACGAAGAAGAC
CATAAAGTTCCTGCAGTGTGTCAGTGATGTGCAAGCTGAATTAGTGCGAAGTGTAC
ACTGTGAAAGCACGTGCTTTTGGCTTATTATGAGAAAACGAAATCTTTAAATT!
CAGTTTATGTGTCTTAGGTCCAGTTTACTTTGATTTGACTACTCAGTTCTTCTGACCCCA
CCTAGTATGTATGTATATGTGTGTGTATGTGTGTGTATGTCTGTATGTATATACATACA
TATACACACACATTGTATACATATGCTATATATACAGTATGTGTATATATATACTATAT
ATGAATATATGAATATATATATTCAATTAGTTAATAGTACATTTAAGCCAAATATCCAA
CATAAGCACACTATGTAAGTATCTATCTGGAAAGACCTATATAGAATTGAGATCAACA
TTTCATGAGTTAGAAACAAAGGATTTTATAATTAATATTACTTAAAGTCTAAAGTACCCA
TATATTTAAATTAGATATGCAATTTTCCCTCTTGGCAAAGAAAGACAAAAATCTTGTG
TTTAGAGATGATGTAGATTGTCAATTTTGCCTTTCCTTCTGAGTACTTGTTTTAAACAAC
AACAAAAAAGACTAGTTTAAAGAAAAGGATTGTCCAGTATTTTCTGCTTTGTTAAG
TCTAATTTTACTGTTAAACAGAGAGCAGAATCACTGGAGTACTGGGGGGGTTTTTGT
GTTTTTTTTTTTTCTTTTCTGTTTTTTTCGGAGCTGGGGAGCGAACCCAGGGCCTTGCG
CTCACTAGGCAAGCACTCTACCGCTGAGCTAAATCCCCAACCCCTGGAGTATCTGTTTT
AAAAGAAAGCCAGGACCGTTATGATGGCCATACCCAGGGTACATAGTGAAAACAACA
GAGACCAAGCAATGAGAGTGTGAGAGTACCAATCCACCAGTACTGCTGCCGGACATG
GCAGCTGCCTGTGCTTTTCTGAAGAGTCATAGTGTATGCTAAGTCTAGAACCATTACTT
AGTAAAGAGGCTATGACTTTTATTTGGGCCTGACAATTTTAGTGGTGTGGTCATAGTCT
ATTCTGTATTTGTAAGCTTTATTTTAAATTACTGTGTTGATTTAGGAAC!
ACAAGAAATGTTTTTATTTTAAATTATGAGTGTATATAAGGTTTTTCTAGATATGCACAGA
!
CTACAATAATAGACTCCCATGGAGATACCACTTCAGCCTTAACAGTCAGGGAGAAGGA
GCCTCACTTTATCACCGCACTCACCTGCTCTCCACTGATCTGTTGTTACTGCGGTGTG
GAGGTTACACGCATGCAGGTCTTACACATGATGGGTAGGCCCCGACCAAGTGAGCC
TCTCCCAGCCTTGCTGTTTTCGTTTTTTTATTTAATCTTACATGTATGGGTGTTTTGCATC
CAGGCATGTCATGCCTGTGTCCACAGAAGCCAGAAAGGGTATCAGATTCCCTAAAACT
GGAGTTCTCGATGATCGTGAGCGAGCCATTGTGGGTGCTGGGAACTGAAGCTGGGTCC
TCTACAAGAGCAGCCAGCGCTCTTAACCATTGAGCCACTATCTGCCCTGTGTTGTTTT
ATTTATTTATTTATTTATTTATTTATTTATTTATTTATTTATTTATTTATTTATTTAT
TGGTCTTTTTTTTTTGGACTGGGGACCGAAGCCAGGGCCTTGCACTTCCTAGGCAAGCG
CTCTACCACTGAGCTAAATCCCCAACCCCTTGTTTTATTTTAAAGCAAACGAGATACA
TAATTTCAACCATGATAATTTAAGATTATCTTGAACCTCTAAGGAAATGTATATACTAA
GCTATTATAGTTTTTATTTTCCCTAATTCAGTGGCATAATACCTTACCTTGAGTCGTTTA
CTACTTCTTTGGTTTCTAAAACTCTACTGCTAAATTACAATGTAAAAACATAGGGCT
CGTATATACTGTAGAGTGCTGTAGATGTCCTCGTCATCAACTATGCAATAACAGTCTGA
TCGACACATTTTCAAGGAGCGATCACTCTTGGTGTGCTTCTTTAAATACTTTTCAAGGCT
TAGGATGTGCAAAGCAGGAAGACCGTGGGTGTAAATGTTTACTTATTTCTTTGAGAGT
GTTAGTAAGTCTTTTCTAAATTGCTTTTCTCTTCAAATATATCGTT!
AACTTAAATGATAATTATCTTTGAGGTAAACAGAAGCTCATTGACAACTAAAGTGA
CTTTTTAGGGCATTCTTTGAGATCATAGTCTTATATCTGGGGACTAAAATGTCATTAGA
CCCTAATAGACTAACTTGTATGTTTGTGTGGGAAACGTTTTCTCTCTCATTCAAGGT

Figure 34c

AACTGTTTGCTGCCTGTTGTTACTTGTGTAGCATTCTAGAAAATGGCTAGGTTTTTTATA
AGATTTAAGACAATAGAAGTAGTTTTATATTATTATAGTTCTGTTGGAATGTGATCCTG
AAATTATTACTGAAAATTAGAATTTTTATTTTCGCTAATGACAACCTTGACTCTCAGAGA
TGCAGTGTAATTGATACCTCATCTTTCCGAGAGTTCAGAGCACAGGGCGGCAGTATG
TGAAGCTGCTTTTGCACTGACGCATTTTGATAAGTTTGGCTACTGTAATGGTAAAAGGC
TCCTCAGGCACTGACTGCATTTGGGTTCTTCCGATGGGGGATGATCCGTTCTCGTGGTG
CTGCTGGACTTATGCATTTTGAGGTAAGTGCATGTATCTTCCCACTGCTTGACATTTTC
TCTGATCTGTGTGTTTGACCAACTCATTAAAAGAAATATGCAGAAATATCTTCTAATT
CGTTGATCTTCGCTGTATGACAGTTATAATATTAAACACTTGGGTTGATCAAAAAAAA
AAAAAAAAAAAAAAAAAAAA

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Figure 35

MGEQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAIINSTITP
NMTFTKTSQKFGQWADSRANTVYGLGFSSEHLSKFAEKFQEFKEAARLAKEKSQEKME
LTSTPSQESAGGDLQSPLTPESINGTDDERTPDVTQNSEPRAEPAQNALPFSHRYTFNSAIMI
K*

CTAGTGGATCCCCCGGGCTGCAGGAATTCTGCGGCCGCAACACCGCACTGTGGTGGAC
AGTGAGGGCCGGAGAGAGACCACAGTGACCCATCAAGAAGCCCATGACAGTTCCAGA
AGTGATCCAGATCCTCCAAGATCTTCAGCTTTGGATGATCCCTTTTCCATCCTGGACCT
GCTTCTAGGACGTTGGTTTCGGTCCCGATAGCTTTCTTGAACCTCAGAGGCCTTCAGGT
CCTTCCCACCCCCTCCCTCCCTGTTGCCATTGCCAATAAGCATAGCTTTTGCTGTCATC
CTGGGGTCTTAAATGTGTGGAACCCCCCAGGGACCTGGTGTGGTGAAATTTGAACCC
ACCGCCTTAGCCCAAAGGCCGAGTAACCTGGCTGCTTGAGTGTCTGGAAGACGTGAG
CGAAATGATCAGCGAACTCATTTTTATCAGACTCGCTGAAGCTGGCTTTTTCGTTTTT
CTACACGTACACTAATTTTTATGGAATAGTTAAAGTGCTATATTCTCCGCGCAACCTTT
CAAATTCAAATGTTTGAACGTTTTGGTGTGACGCGGAGTGAAATCATTTTACCGACAA
GAACTAACTGAATTGTCTGCCTCGTTGAGTTGCCTCCGGAAGATCTCGGGGGTGGAA
AAAGCAACTGCAAAATAACAGACGGAGAAAATTCCTTGGAAAGTTATTTCTGTAGCATA
AGAGCAGAACTTAAGAGCAAGTTTTATTGGGCAAAATGGGGGAACAACCTATCTTC
AGCACTCGAGCTCATGTCTTCCAGATCGACCCAAACACAAAGAAGAACTGGGTACCCA
CCAGCAAGCATGCAGTACTGTGTCTTATTTCTATGACAGCACAAAGGAATGTGTATAG
GATAATCAGTCTAGACGGCTCAAAGGCAATAATAAATAGCACCATCACTCCAAACATG
ACATTTACTAAAACATCTCAAAAGTTTGGCCAATGGGCTGATAGCCGGGCAAACTG
TTTATGGACTGGGATTCTCCTCTGAGCATCATCTCTCAAAATTTGCAGAAAAGTTTCAG
GAATTTAAAGAAGCTGCTCGGCTGGCAAAGGAGAAGTCGCAGGAGAAGATGGAAGT
ACCAGTACCCCTTCACAGGAATCAGCAGGAGGAGATCTTCAGTCTCCTTTAACACCAG
AAAGTATCAATGGGACAGATGATGAGAGAACACCCGATGTGACACAGAACTCAGAGC
CAAGGGCTGAGCCAGCTCAGAATGCATTGCCATTTTACATAGTTTACGCCATCAGCAA
ACACTGGGAGGCTGAACTAGCCACGCTCAAGGGGAACAATGCCAAGCTCACCCGAGC
GCTGCTGGAGTCCACTGCCAACGTGAAGCAGTGGAAGCAACAGCTGGCTGCCTACCAG
GAGGAGGCAGAGCGGCTGCACAAGCGGCTACGGAGCTGGAATGTGTTAGTAGTCAA
GCAAACGCGGTGCACAGCCACAAGACAGAGCTGAGTCAGACAGTGCAGGAGCTGGAA
GAGACCCTAAAAGTAAAGGAAGAGGAAATAGAAAAGATTAACAAGAAATTGATAAC
GCCAGAGAAGTTCAAGAACAGAGGGACTCTTTGACTCAGAAACTACAGGAAGTTGAG
ATTGAAATAAAGACCTGGAGGGGACGCTGTCTCGAGCTGGAGCAGCGCCTGGAGAAG
AGCCAGAGCGAGCAGGACGCTTTCCGCAGTAACCTGAAGACTCTCCTAGAGATTCTGG
ACGGGAAAAATTTTGAACAAACAGAAATTGCGGGATAATTTGGCCAAGCTACTAGAATG
CAGCTAAAGAAAGTGAAATTTTCAAGTCCAATAGATGAAGAGATACTGTCTGTCTTCGT
AGGACTGTTTGGGCTCTGTACCAAGATTGCACAAAATTTTTTGAATATCATTCTCCAG
AAGGAGGGTGTTTTGAATTTGGAATTGTATTTTCAAGTATAAATTTTAGAATTTAGCT
TATAGCTAGTTGGGGGAAAAAAGACATGAAAACTTGAACCACAAATTACCTCCATG
TACATTGGCCATAGTTACAATGGGAGAATTAACAATGTCTGGGTCCCTTCTCCTTTTTC
TGTTCAACACAGTGAAGATTATCTGCTTTTTTAAATTTATTTACGATATCTACAGCTGTG
TTTTGTGTAACAACTTAGTAATGGAAGCCCTGTCTTTGTTGTTATCTGAATAATTTCTCA
GGATATTTTTTTGCTGCTGAGAAAGGGCCATTACCAATTAATCCTTGCCAGGAGTTGGG
GAGCTATGTCTCTAATTGGAATCACTATAACTGGGTGTCTGGAGTTCTTCCCTTTTCGT
ACTGAGAGTGTCTCACTCTAGTACTCCTCTGGTACACTCCGTGTTCTCCAATCTTGTC
TGTTGTACTTTACTTTTCCATATTGACTCCATGTATTTATGAGAAGATATTATCTCCCAT
TTTATTATACATTTTGAAGCCAACTAAACAAAGGCAGCTGAGTCCTTCAGATATTTTC
TTTTTAAATTTATAGTAAATTTGACACAGAACTGAAATTCAGCAGTCCGTCTTTGACGG
TTTAGTCTAGCAATGTTAAGGATATTTAGAGAAAATATGCAGTTACGTTTATTTATATA
TTTGGCAAGAAATTTTTCTGGATGATCAATGCTTTTCAATTTATGATAAATAATGGTT
AGGGGGCGCTGTTTATTATAGATAATTTTAAAGGTATATAGCTGTTTTCAAGGAGGTCCA
CTTCCGTCTAGCAGCCAAGCAGAGGACTGTATCTAAATCGTGATCGTGGCAGATGGGT
CTTCATAGAAACCATGTCTTTATTCAAACCTTCATAGGGCAATATTTGAACTGTTACCT
AGGCATTTCAAAACAGGAAATACCGTCAACAGACTCTTCTCCAAGAGCAGGTTTTACT

Figure 36b

GTTGTTTTGATGTAATTTTAAGACATTTAGCAAACATGCATTTCTTTATATGATACATTT
CTTTCACAAAACAATTTAAAAGTAAGCCACGTGCTGTCTGCTCTGCCCCGGGTAGGAAT
TGCATCAGAATACATATATCTTGCTGTACAATGCCTGTGATATTGAAGAGGGTTCTTTT
CATGTATGCTTGAGTATCTAACTCTGGAGTCAATGAATGCACTGACTTTTTTTTGTTCG
TACCCCAAATGATTGAATTGTTAAGTACAAATTAAGCAGATTAACCTCAITTTTTTCACTC
ATAAACAGATTCTTAGTACTAGTTTTGTTTTATTTATGTGTATGTATGTAAATACATA
CATATTAATTTATATTAGAGTGAAAAATAAATTGTTTGTTTCTAACATTAACAAAAA
AAAAAA

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Figure 37

MGEQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAIINSTITP
NMTFTKTSQKFGQWADSRANTVYGLGFSSEHHLSKFAEKQEFKEAARLAKEKSQEKME
LTSTPSQESAGGDLQSPLTPESINGTDDERTPDVTQNSEPRAEPAQNALPFSHSSAISKHWE
AELATLKGNNAKLTAALLESTANVKQWKQQLAAYQEEAERLHKRVTELECVSSQANAV
HSHKTELSQTVQELEETLKVKEEEIERLKQEIDNARELQEQRDSLQKLQVEIRNKDLEGQ
LSELEQRLEKSQSEQDAFRSNLKTLLLEILDGKIFELTEL RDNLAKLLECS*

Figure 38b

AGACTCTTCTCCAAGAGCAGGTTTTACTGTTGTTTTGATGTAATTTTAAGACATTTAGC
AAACATGCATTTCTTTATATGATACATTTCTTTCACAAAACAATTTAAAAGFAAGCCAC
GTGCTGTCTGCTCTGCCCCGGGTAGGAATTGCATCAGAATACATATATCTTGCTGTACAA
TGCCTGTGATATTGAAGAGGGTTCTTTTCATGTATGCTTGAGTATCTAACTCTGGAGTC
AATGAATGCACTGACTTTTTTTTTTGTTCGTACCCCAAATGATTGAATTGTTAAGTACA
AATTAAGCAGATTAACTCATTTTTTCACTCATAAACAGATTCTTAGTACTAGTTTTGTTT
TATATTTATGTGTATGTATGTAAATACATACATATTAATTTATATTAGAGTGAAAAATA
AATTGTTTGTCTAACATTAGTTTCTACAGTAAGGTGTCTCTGAAACATGTGTGTCAG
ACACTTAGCCACCATGCATTCTATGTGCTACCCCATCATGCCAGTCACCTCCATCGACG
TTAGGGTATTTTCCTTACCTGTCTATTATAAAGAGAATAACTTAGGTACACATGCTCAG
AGCCGAGATATTTCTCTGATAAATCAGGTAATAAAATCTATTTGATGGGTAGAATTTTG
AAAACAGACATGATTTTATCTATGAGTTTCTGAATATCAAAGAACACCAGGTTTTCATT
TAAATAGAGGTCTAACACTAGGGATCAGGGAATTTAGTTATGAAGAGTTGAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAA

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Figure 39

MGEQPIFSTRAHVFQIDPNTKKNWVPTSKHAVTVSYFYDSTRNVYRIISLDGSKAINSTITP
NMTFTKTSQKFGQWADSRANTVYGLGFSSEHLSKFAEKFQEFKEAARLAKEKSQEKME
LTSTPSQESAGGDLQSPLTPESINGTDDERTPDVTQNSEPAEPAQNALPFSSAGDRTQGL
SHASSAISKHWEAELATLKGNNAKLTAALLESTANVKQWKQQLAAYQEEAERLHKRVTE
LECVSSQANAVHSHKTELSQTVQELEETLKVKEEEIERLKQEIDNARELQEQRDSLTKLQ
EVEIRNKDLEGQLSELEQRLEKSQSEQDAFRSNLKTLLILDGKIFELTEL RDNLAKLLECS*
R

Figure 40a

R-SHANK3A(GENBANK)(nn)

CTCTAGAACTAGTGGATCCCCCGGGCTGCAGGATTCTGCGGCCGCGCTAAACCGTGCC
GCCGTCGCCGCCGCGCTGCGCCTGCGGAGCCCCCGAGCCGCTGTCCCCCGCGCTGG
CCCCGGCCCCCGCCCCATACGGCCCCCTCCCGCAGTAGCGCGGTGCGCGGGACTCTGG
CGGGGGGTACGGGGGGGCCAGGGCGCCGCGCGGAGTCCCCGTGCGCTCCTCTCTCCGC
CGGGAACAGTCCGGGGCCCCGGCGCTAGCACCGGGATGGACGGCCCCGGGGCCAGCGC
CGTGGTCGTGCGCGTCGGCATCCCGGACCTGCAACAAACGAAGTGCCTGCGTCTGGAT
CCAACCGCGCCCGTGTGGGCCGCCAAGCAGCGTGTGCTCTGCGCCCTCAACCACAGCC
TTCAGGACGCGCTCAACTACGGGCTATTCCAGCCTCCCTCCCGGGGTGCGCGCCGGCAA
GTTCTTGATGAAGAGCGGCTCTTACAGGACTACCCGCCTAACCTGGACACGCCCCTG
CCCTATCTGGAGTTTCGATACAAGCGGAGAGTTTATGCCCAGAACCTCATAGATGACA
AGCAGTTTGAAAGCTGCACACAAAGGCAAACCTGAAGAAAGTTCATGGACTATGTCCA
GCTACACAGCACAGACAAGGTGGCCCCGCTGCTGGACAAGGGGCTGGACCCCAATTTC
CATGACCCTGACTCAGGAGAGTGCCCTCTGAGCCTTGACAGCACAGTTGGACAACGCCA
CTGACCTCCTGAAGGTTCTTCGCAATGGCGGTGCTCATCTGGACTTCCGAACCCGAGAT
GGGCTAACCGCTGTCCACTGCGCCACCCGACAGCGGAATGCGGGAGCATTGACGACCC
TGCTGGACCTGGGGGCTTCACCTGACTACAAGGACAGCCGCGGCCTGACGCCCCGTGA
CCATAGTGCCCTAGGGGGCGGGGATGCCCTCTGCTGTGAGCTGCTTCTCCATGATCAC
GCACA!
GTTGGGGACCACTGACGAGAATGGCTGGCAGGAGATCCATCAGGCCTGTGCTTTGGG
CATGTACAGCACTTGGAGCACCTGCTGTTCTATGGGGCCAACATGGGTGCCCAGAACG
CCTCGGGAACACACGCTTGACATCTGTGCCCTCTATAACCAGGAGAGCTGTGCCCG
CGTCTGTCTTTCCGTGGTGCCAACAAGGACGTCCGCAATTACAACAGCCAGACAGCC
TTCCAGGTGGCCATTATTGCAGGGAACCTTTGAGCTTGCCGAGGTAATCAAGACCCACA
AAGACTCGGATGTGCTACCATTCAGGGAAACCCCCAGCTATGCAAAGCGACGACGTCT
GGCTGGCCCGAGTGCTTGGCATCCCTCGGCCCTTACAGCGCTCAGCCAGTGATATC
AACCTGAAGGGTGACCAGCCCGCAGCTTCTCCCGGGCCCACTCTCCGAAGCCTCCCTC
ACCAACTGCTGCTCCAGAGGCTTCAGGAGGAGAAAGACCGGGACAGGGATGGTGAGC
AGGAGAACGACATCAGCGGTCCCTCAGCAGGCAGGGGCGGCCACAGCAAGATCAGCC
CCAGCGGGCCCGGCGGATCCGGCCCCGCGCCCGGCCCGGCCCGGCGTCTCCCGCGCC
CCCCGCGCCCGCCCGCCCGGGGCCCCGAAGCGGAACTTTACAGTGCCGTCCCCGCGCCG
AAGTTCATCGCTGTGAAGGCGCACAGCCCGCAGGGCGAGGGCGAGATCCCGCTGCAC
CGCGGCGAGGCCGTGAAGGTGCTCAGCATTGGGGAGGGCGGTTTCTGGGAGGGAACC
GTGAAGGGCCGTACAGGCTGGTTCCAGCTGACTGTGTGGAGGAAGTGCAGATGCGAC
AGTATGACACACGGCATGAACTCGAGAGGACCGGACGAAGCGTCTTTCCGCCACTA
CACTGTGGGTTCTATGACAGCCTCACTTCACACAGTGATTATGTCATTGATGATAAGG
TGGCTATC!
CTGCAAAAACGGGACCATGAGGGTTTTGGCTTTGTTCTCCGGGGAGCCAAAGCAGAGA
C!
CCCCATTGAGGAGTTTACACCCACACCTGCCTTCCCTGCGCTCCAGTACCTTGAGTCTG
TAGATGTGGAAGGTGTGGCCTGGAAGGCTGGGCTTCGCACTGGGGACTTCCTCATTGA
GGTAAACGGAGTGAACGTCGTGAAGGTTGGACACAAGCAAGTGGTGGGTCTCATCCGT
CAGGGTGGCAACCGTCTGGTCATGAAGGTTGTGTCTGTTACCAGGAAGCCAGAGGAGG
ATAGTGCTCGGCGCAGAGCCCCACCACTCCCAAGAGGGCCCCCAGCACCGCTGAC
CCTGCGGTCCAAGTCCATGACGGCTGAGCTCGAGGAACCTCGCTTCCATTCCGAGAAGG

Figure 40b

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AAAGGGGAGAAGTTGGATGAGATCCTGGCGGTTGCTGCGGAACCAACGCTGAGGCCA
GACATTGCAGACGCTGATTCCAGGGCAGCCACTGTCAAGCAGCGGCCACCAGCCGGA
GGATTACCCCTGCCGAGATCAGCTCATTGTTTGAGCGACAGGGCCTCCCGGGCCCA
GAAGCTGCCGGGCTCTCTGCGGAAGGGGATTCCACGGACCAAATCTGTAGGGGAGGA
TGAGAAGCTGGCATCCCTACTGGAAGGGCGTTTCCCACGCAGCACATCAATGCAAGAC
ACAGTGCCTGAAGGCCGAGGCATTCCGCCCCACCGCAGACCGCCCCGCCACCCCCAC
CCGCGCCCTACTACTTCGACTCCGGGCCACCCCCACCTTCTCACCACCGCCACCACCA
CCGGGCCGGGCTATGACACTGTGCGCTCCAGCTTCAAGCCAGGCCTGGAGGCTCGTC
TGGGTGCAGGGGCAGCTGGCCTGTATGATTCTGGCACACCTCTGGGCCCCGCTGCCCTA
CCCTGAGCGCCAGAAGCGTGCACGCTCCATGATCATATTGCAGGACTCTGCGCCAGAA
GTGGGCGATGTACCCCGGCTGCGCCTGCAGCCACACCGCCTGAGCGCCCCAAGCGCC
GCCT!
CGGCCGTGAGGCCCTGATAGTCCCTATGCCAACCTGGGCGCCTTCAGTGCCAGCCTCTT
TGCTCCGTCGAAACCGCAGCGCCGCAAGAGTCCGCTGGTGAAGCAGCTTCAGGTGGAG
GACGCTCAGGAGCGCGCGCGCTTGGCCGTGGGTAGCCCCGGGACCAAGTGGGTGGAAGC
TTTGACAGAGAACCCTCCCCAACGCACCGCGGGCCCCGACCGGGCGGCCTTGACTACA
GCTCTGGAGAAGGCCTGGGGCTCACCTTTGGCGGCCCTAGCCCTGGCCAGTCAAGGA
GCGGCGCCTGGAGGAGCGACGCGCTTCCACTGTGTTCTGTCTGTGGGTGCCATCGAG
GGCAACCTCCCAGCGCGGATCTGCCATCCCTACAACCCTCCCGCTCCATTGATGAGC
GCCTCCTGGGACAGGCGCCACCCTGGCCGAGATTTGCTGCTCCCTCCCTGTCTCT
GCTCTGAAGCCATTGGTTCGGTGGTCCCAACCTTGGGCCCCCAAGCTCCACCTTCATCCA
TCCTCTTACTGGCAAACCCTTGATCCTAGCTCACCCTAGCTCTTGCTCTGGCTGCCC
GAGAGCGGGCTCTGGCCTCGCAAACACCTTCCCGGTCCCCACACCCGTGCACAGTCC
TGATGCTGACCGCCCTGGACCCCTCTTTGTGGATGTGCAAACCCGAGACTCCGAGAGA
GGACCCTTGGCCTCCCCAGCCTTCTCCCCTCGGAGTCCAGCCTGGATTCCAGTGCCTGC
TCGAGAGAGGAGAGAGAAGCCCACTCGGGAAGAGCGGAAGTCACCAGAGGACAAGA
AATCCATGATCCTCAGCGTCTTGACACGTCCTTGCAACGGCCAGCTGGCCTCATTGTT
GTGCATGCCACCAGCAATGGACAGGAGCCCAACAGGCTGGGGGCTGAAGAGGAGCGC
CCGGGTACTCCGAGCTGGCCCCAACCCCCATGCAGGCAGCAGCTGTGGCAGAGCCCA
TGC!
CAAGCCCACGAGCCCAACCCCCTGGCAACATCCCAGCAGATCCCGGGCCAAGCCAAG
GC!
AACTCAGAGGAGGAGCCAAAGCTGGTATTGCTGTGAACCTGCCACCTGCTCAACTGT
CCTCCAACGATGAGGAGACCAGAGAGGAGCTGGCCCGCATTGGGCTAGTGCCACCCCC
TGAAGAGTTTGCCAATGGGATCCTGCTGGCCACCCCCACCCCAGGACCGGGCCCCCTG
CCCACCACGGTACCCAGCCCGGCCTCAGGGAAGCCCAGCAGCGAGCTGCCCCCTGCCC
CGGAGTCTGCAGCTGACTCTGGAGTAGAGGAGGCCGACACTCGAAGCTCCAGTGACCC
CCACCTGGAGACCACAAGCACCATTTCACAGTGTCCAGCATGTCCACCCTGAGCTCG
GAGAGTGGAGAACTCACTGACACCCACACCTCCTTTGCCGATGGACACACTTTTCTACT
CGAGAAGCCACCAAGTGCCTCCCAAGCCCAAACTCAAGTCCCCGCTGGGGAAGGGGCC
GGTGACCTTCAGGGGCCCCGCTGCTGAAGCAATCCTCGGACAGTGAGCTCATGGCCAG
CAGCACCATGCCACCTCTACTGGGTTGACTTCTGCTGCTGGGCCTGCCCGCCCTCGTA
CCTCTTCCAGAGAAGGTCCAAGCTGTGGGGGACCCCGTGGAGAGTCCGGGGCTCCCT
GGCCCTGAGGATGACAAACCAACTGTGATCAGTGAGCTCAGCTCCCGTCTGCAGCAGC
TGAATAAGAACTCGCTCCTTGGGGGAGGAACCAAGTTGGTGGCCTGGGTAGCCTGCT
GGACCCTGCTAAGAAGTCGCCCATTGCAGCAGCTCGCTGCGCGGTGGTCCCGAGTGCC

Figure 40c

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GGCTGGCTCTTCAGCAGCCTCGGTGAGCTGAGCACCATCTCAGCGCAGCGCAGCCCCG
GGGGCCCCGGGCGGAGGGGGCCTCCTACTCGGTGCGGCCAGCGGCCGGTACCCCGTGGC
GAGACGAGCCCCGAGCCAGTGAAACCCGCATCGCTGGAGCGGGTGGAGGGGCTGGG
GGCGG!
GCGTGGGAGGCGGGGGCGGCCCTTCGGCCTCACGCCTCCCACCATCCTCAAGTCGTC
CAGCCTCTCCATCCCCGACGAACCCAAGGAAGTGCCTTCGTGGTGCGAAGTGCGAGT
GCGCGCAGCCGCTCCCCCTCACCATCTCCGCTGCCCTCGCCTTCTCCTGGCTCTGGCCC
CAGTGCCGGCCCCGCGTCGGCCATTCAACAGAAGCCCCTGCAGCTTTGGAGCAAGTTC
GATGTGGGCGACTGGCTGGAGAGCATCCACTTAGGCGAGCACCGAGACCGCTTCGAG
GACCATGAGATCGAAGGCGCACACCTGCCTGCGCTCACCAAGGAAGACTTCGTGGAGC
TGGGAGTACACGCGTTGGCCACCGCATGAACATCGAGCGTGCGCTCAGGCAGCTGGA
TGGCAGCTGACGCCCCCTCTCCCTCTCCTGTTCTGCTGCGCCCTGCCGGCAGGGCCCCC
ACCCCTACTCCAGGCCGCGAGGCTCGGCTCGCCCCCTACCACGGCGCCCGGGCCAGGAA
TGTTGCATGAATCGTCCTGTTTGCTGTTGCTTGGAGACTTGCCCTGTACATTGCTTAGTG
CCCTCCCCTGCCGCTGAACCCCAACCAGCACACAGTAAGGGCGCGGACCAGGGGGGCT
GGGTGGAAGGGGGTTGGGGCAGGGTGCTCTGGCCTGACCACCTCCTCCACAGCTCCTG
GTGGCCATTCTTCCAGAGGGGGAACCTAGTCCAGCATGCGAGGTACGGACACGCCTTG
GTGACTCGGGGGGAGGGGGGAGACATTGGGGTTCTCGATAGGGGCCAAGGAGCCCCC
TGTTTTACATATTTTAATCCACTCTATATTTGGAAAGAGAAAAGGAACAAATATCTTG
TCCGTAACAGTTCGCGCCCTCTTCCCCTCAAGTCCTCTCGCTGGTCCCGCCACAGCTAC
CCAGTCTTCCATCTCCGGCCCCCTCACTGCCACCCCATATAGGGCAGGGGACACTCCAG
C!
TGGCCTGGGGTTAGCCAGGGTCCTGGCAGCCCACCCTGGGGACCCCGGCTCAGCCCCC
T!
TCCCTCGCTGAGCTATAGTATGCCCCACCCACCCTTTAGGTGCTGCTCAGGGGGACGG
GTGGCAGGCATTGCCTGCTGGGCACTAGCAGGGCCAGGTGGCCTGGGAGATTATTGCC
CTGGGGCTGGGCCCCGGTAACCCAACCCAGCCATCATCTTACAGGGTCTCTCCCAA
AGGAGGGGTCTAACCTTTCCCACTTCTTGGGCAACTACAGCAGAGAAGCCTCCCTGC
CTCGCGCCCCAAAGACTCCCCAATTCCTGCCCTGTGTGTGTGCACCACATGTGTGTGTG
CACGCCTGCGTGCTTGTGAAAATTGGGTGTGGCTGAGCGCATGGGTGCCCTGTATGTG
CTTGATTGTGGAGTGGTCCCCAGGGGCTGTTCTGGATGGGTGGGAGGTTGAGGAAGCT
TGCACAGGGGTGCATGCATGGGTGTGTGCCTGTGAAAGGGCCCTGTCTTCTCCAAAGA
AAGGCTGTCTGCTCTTGGGTCTGCTGTTTTCTCAGCCTGTTCTCCCTGAACCTCACCC
AGCTTAAGCAGGGGTCTTGGTGAATCCTTTCAGCTTTGGGAGGCCTCAAGGGCTCCC
GTGCAGGCAGCACCCCTTTGGGCTTCTAAGGGAATTGTGGGGACCACTAAAATCAGGC
CACAACAGCCCTTGGAGAGAGGCCAAAGACTCCTGAGGGTACCCTGGCCCCCTTACTG
TGACTCCTCACAATTACGAATGACCTGTGGGGCGGGGGGCCTTGGGGCATTTTTAAC
ATAGGGTTTGGAGTCTGGAATAAGCTCCATCCACGTCACTCACAAGTTTCTGTTTCTAT
TTCTAGCTTTTTTTAATAAAATATATATATATATATAAAAGACAGAAAACAGGTG
TTTTATGGCCAGGGGCTTGGCACGCCGGTCTGTGCCACCCGCCCGCCCCACCCCTG
GCCACCGGCCCATTCCTTAGACACAGAGTCACGCCCACTAACCTCTTACCAACA!
GAGCAGGTACACACACAGCAGCGGTCACTGTAACAGACTGCCACATACACAGTCTCA
CATTTACCTGTGGGTTTTTGGTTCTGTTCAGTTTGGGTTTTTAACTTTACAGGGTCAGTT
CCGCTTCATCCCCCTTTTGTATGGAGTTCATCTCGGGGCTTTCAACCCCTGCTCCAGT
CCTGAGGCCTCCTGACCCTGACGTTGTGATACACCCACAGAGATCTATGTTTCTTATA

Figure 40d

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TTATTATTATTAATAATAATTATTATAATATTATGTAATAAATTTATAAGAAATGAAA
AAAAAAAAAAAAAAAA

r-shan2

Figure 41

MDGPGASAVVVRVGIPDLQQTCLRLDPTAPVWAAKQRVLCALNHSIQDALNYGLFQPP
SRGRAGKFLDEERLLQDYPPNLDTPLPYLEFRYKRRVYAQNLIIDDKQFAKLHTKANLKKF
MDYVQLHSTDVKVARLLDKGLDPNFHDPDSGECPLSLAAQLDNATDLLKVLNRNGGAHLDF
RTRDGLTAVHCATRQRNAGALTLLDLGASPDYKDSRGLTPLYHSALGGGDALCCCELLH
DHAQLGTTDENGWQEIHQACRFHVQHLEHLLFYGANMGAQNASGNTALHICALYNQES
CARVLLFRGANKDVRNYSQTAQVAVIAGNFELAEVIKTHKDSDVVPFRETSPYAKRRRL
AGPSGLASPRPLQRSASDINLKGDQPAASPGPTLRSLPHQLLLQRLQEEKDRDRDGEQENDI
SGPSAGRGGHISKISPSGPGSGPAPGPGPASPAPPAPPPRGPKRKLYSAPVGRKFIAVKAHS
PQGEGEIPLHRGEAVKVLISGEGGFWEQTVKGRTGWFPADCVVEVQMRQYDTRHETRED
RTKRLFRHYTVGSYDSLTSYSDYVIDDKVAILQKRDHEGFGFVLRGAKAETPIEEFTPTPAF
PALQYLESVDVEGVAWKAGLRTGDFLIEVNGVNVVKVGHKQVVGILRQGGNRLVMKV
SVTRKPEEDSARRRAPPKRAPSTTLRLSKSMTAELEELASIRRRKGEKLDEILAVAAEP
TLRPDIADADSRAATVKQRPTSRRITPAEISSLFERQGLPGPEKLPGLSRKGIPRTKSVGEDE
KLASLLEGRFPRSTSMQDTVREGRIPPPPQTAPPPPPAPYYFDSGPPPTFSPPPPPPGRAYD
TVRSSFKPGLEARLGAGAAGLYDSGTPLGPLYPERQKRARSMIILQDSAPVGDVPRPAP
AATPPERPKRRPRPSGPDSPYANLGAFSASLFAPSKPQRRKSPLVKQLQVEDAQERAALAV
GSPGPVGGSFAREPSPTHRGPRPGGLDYSSGEGGLTGGGSPGPVKERRLEERRRSTVFLS
VGAIEGNPPSADLPSLQPSRSIDERLLGTGATTGRDLLLSPVSAKPLVGGPNLGPSSSTFIH
PLTGKPLDPSSPLALALAAARERLASQTPSRSTPVHSPDADRPGLFVDVQTRDSEGRPLA
SPAFSPRPAWIPVPARREAEKPTREERKSPEDKKSMILSVLDTSLQRPAGLIVVHATSNGQ
EPNRLGAEERPGTPELAPTPMQAAA VAEPMPSRAQPPGNIPADGPSQGNSEEPKLVF
AVNLPPAQLSSNDEETREELARIGLVPPPEEFANGILLATPPPGPGPLPTTVPSASGKPSSEL
PPAPESAADSGVEEADTRSSSDPHLETTSTISTVSSMSTLSSESGETDTHTSFADGHTFLE
KPPVPPKPKLSPLGKGPVTFRGPLLKQSSSELMAQQHHATSTGLTSAAGPARPRYLFQR
RSKLWGDVPESRGLPGPEDDKPTVISELSSRLQQLNKDTRSLGEEPVGGLGSLLDPAKKSPI
AAARCAVVPSAGWLFSSLGELSTISAQRSPGGPGGGASYSVRPSGRYPVARRAPSPVKPAS
LERVEGLGAGVGGAGRPFGLTPPTILKSSSLIPHEPKEVRFVVRASARSRSPPSPSPSP
SGSPSAGPRRPFQKPLQLWSKFDVGDWLESIHLEHRDRFEDHEIEGAHLPALTKEDFVE
LGVTRVGHMNIERALRQLDGS

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Figure 42

ATGTCCACAGCCAGGGAGCAGCCAATCTTCAGCACACGGGCGCACGTGTTCCAAATTG
ACCCAGCCACCAAGCGAACTGGATCCCAGCGGGCAAGCACGCACTCACTGTCTCCTA
TTTCTACGATGCCACCCGCAATGTGTACCGCATCATCAGCATCGGAGGCGCCAAGGCC
ATCATCAACAGCACTGTCACTCCCAACATGACCTTCACCAAACTTCCCAGAAGTTCG
GGCAGTGGGCGGACAGTCGCGCCAACACAGTCTATGGCCTGGGCTTGCCTCTGAACA
GCATCTGACACAGTTTGCCGAGAAGTTCCAGGAAGTGAAGGAAGCAGCCAGGCTGGC
CAGGGAGAAATCTCAGGATGGCTGGGGTGGGCCCCAGTCGGCTCTGGTTGTTGGCAGC
TTTGGGGCTGTTTTTGAGCTTCTCATTGTGTAGAATTTCTAGATCCCCGATTACATTTC
TAAGCGTGA

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Figure 43

MSTAREQPIFSTRAHVFQIDPATKRNWIPAGKHALTVSYFYDATRNVYRIISIGGAKAINST
VTPNMTFTKTSQKFGQWADSRANTVYGLGFASEQHLTQFAEKFQEVKEAARLAREKSQD
GWGGPQSALVVGSGAVFELLIV*

ratina2

Figure 44

CACGCGTCCGGTGTGGTGCACCTTGGCATCTGTAAGCCTTTGGTGGAGGAGGAGAAGG
AGGAGAAGGAGGAACATTTTATTTTCCATTCAAACAACAATGGAGATAACAGTGAGTC
TCCAGAAACCGTTCACGAGATCCACTCATCTTTAATCCTCGAGGCACCCCAGGGATTTA
GAGATGAGCCGTATCTTGAAGAACTCGTGGATGAACCTTTTCTAGATTTGGGAAAGTC
TTTGAGTTCCAACAAAAAGACATGGACAGCAGCTCAGAAGCCTGGGAAATGCATGA
ATTCCTGAGCCCTCGGCTGGAGAGAAGGGGTGAGGAAAGAGAGATGCTTGTTGACGA
GGAGTATGAGATCTACCAAGACCGCCTCCGGGACATGGAAGCACACCCACCACCTCCT
CACATTCGGGAGCCCACCTTCTGCATCTCCCAGGCTGGATCTCCAGGCCGGCCCCCAGT
GGCTGCATGCTGACCTCTCAGGAGGAGAGATACTCGAGTGTACGACACAGAGTCCAT
GATGACTGCTTATCCCCAGGAGATGCAGGACTATAGCTTCAGCACACAGACATGATG
AAAGAAACATTTGGCCTTGACTCCCGGCCGCCCATGCCCTCCTCTGAAGGAAATGGTC
AGCACGGCCGATTTGATGACTTGGAACATCTTCATTCAGCAAGCCACGGCCTGGA
TTTAGGCATGATGACTCCAAGTGACTTGCAAGGCCCTGGCGTGCTTGTAGATCTTCCAG
CTGTCACCCCAAGAAGAGGCTGCGGCCGCTAAGTAAGTAAGACGTCGAGCTCTAAGTA
AGTAACGGCCGCCACCGCGGTGGAGCTTTGGACTTCTTCGCCAGAGG

ratinal

Figure 45

ID rat INADL PRT; 286 AA.
SQ SEQUENCE 286 AA; 31933 MW; 426539 CN;
HASGVVHLGI CKPLVEEEKE EKEEHFIFHS NNNGDNSESP ETVVEIHSSL
ILEAPQGFRD
EPYLEELVDE PFLDLGKSLQ FQKDMDSSE EAWEMHEFLS PRLERRGEER
EMLVDEEYEI
YQDRLRDMEA HPPPPHIREP TSASPRDLQ AGPQWLHADL SGGEILECHD
TESMMTAYPQ
EMQDYSFSTT DMMKETFGDL SRPPMPSSEG NGQHGRFDDL EHLHSLASHG
LDLGMMTPSD
LQPGVLVDL PAVTPRRGCG R*VSKTSSSK *VTAATAVEL WTSSPE

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ratinal

Figure 45

ID rat INADL
SQ SEQUENCE 286 AA; 31933 MW; 426539 CN;
HASGVVHLGI CKPLVEEKE EKEEHFIFHS NNNGDNSESP ETVHEIHSSL
ILEAPQGFRD
EPYLEELVDE PFLDLGKSLQ FQKQDMDSSS EAWEMHEFLS PRLERRGEER
EMLVDEEYEI
YQDRLRDMEA HPPPPHIREP TSASPRDLQ AGPQWLHADL SGGEILECHD
TESMMTAYPQ
EMQDYSFSTT DMMKETFGDL SRPPMPSSSEG NGQHGRFDDL EHLHSLASHG
LDLGMMTPSD
LQGPGLVLDL PAVTPRRGCG R*VSKTSSSK *VTAATAVEL WTSSPE

